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(54) Title: FOOD ADDITIVE COMPRISING PSEUDOMONAS NON-MALTOGENIC EXOAMYLASE VARIANTS

(57) Abstract: We disclose a food additive comprising a PS4 variant polypeptide, in which the PS4 variant polypeptide is derivable from a parent polypeptide having non-maltogenic exoamylase activity, in which the PS4 variant polypeptide comprises substitutions at the following positions: 134, 141, 157, 223, 307 and 334 with reference to the position numbering of a *Pseudomonas saccharophila* exoamylase sequence shown as SEQ ID NO: 1.

WO 2005/003339 A1

FOOD ADDITIVE COMPRISING PSEUDOMONAS NON-MALTOGENIC EXOAMYLASE VARIANTS

FIELD

This invention relates to polypeptides, and nucleic acids encoding these, and their uses as non-maltogenic exoamylases in producing food products. In particular, the polypeptides are derived from polypeptides having non-maltogenic exoamylase activity, in particular, glucan 1,4-alpha-maltotetrahydrolase (EC 3.2.1.60) activity.

SUMMARY

According to a first aspect of the invention, we provide a food additive comprising a PS4 variant polypeptide, in which the PS4 variant polypeptide is derivable from a parent polypeptide having non-maltogenic exoamylase activity, in which the PS4 variant polypeptide comprises substitutions at the following positions: 134, 141, 157, 223, 307 and 334, with reference to the position numbering of a *Pseudomonas saccharophilia* exoamylase sequence shown as SEQ ID NO: 1.

Preferably, the PS4 variant polypeptide comprises one or both of substitutions at positions 121 and 223, preferably G121D and/or G223A. The position 223 substitution may also comprise G223L. More preferably, the PS4 variant polypeptide comprises one or more of: a substitution at position 33, preferably N33, more preferably N33Y, a substitution at position 34, preferably D34, more preferably D34N, a substitution at position 178 and a substitution at position 179.

Preferably, the parent polypeptide comprises a non-maltogenic exoamylase, preferably a glucan 1,4-alpha-maltotetrahydrolase (EC 3.2.1.60). Preferably, the parent polypeptide is or is derivable from *Pseudomonas* species, preferably *Pseudomonas saccharophilia* or *Pseudomonas stutzeri*. In preferred embodiments, the parent polypeptide is a non-maltogenic exoamylase from *Pseudomonas saccharophilia* exoamylase having a sequence shown as SEQ ID NO: 1 or SEQ ID NO: 5. In highly preferred embodiments, the parent polypeptide is a non-maltogenic exoamylase from *Pseudomonas stutzeri* having a sequence shown as SEQ ID NO: 7 or SEQ ID NO: 11.

In highly preferred embodiments, the PS4 variant polypeptide has a higher thermostability compared to the parent polypeptide when tested under the same conditions. Preferably, the half life ($t_{1/2}$), preferably at 60 degrees C, is increased by 15%

or more, preferably 50% or more, most preferably 100% or more, relative to the parent polypeptide.

Alternatively or in addition, the PS4 variant polypeptide preferably has a higher exo-specificity compared to the parent polypeptide when tested under the same conditions.

35 Preferably, it has 10% or more, preferably 20% or more, preferably 50% or more, exo-specificity compared to the parent polypeptide.

In further preferred embodiments, the PS4 variant polypeptide is one in which the position 134 substitution comprises G134R. The position 141 substitution preferably comprises A141P. furthermore, the position 334 substitution preferably comprises S334P.

40 Preferably, the PS4 variant polypeptide is one in which (a) the position 33 substitution comprises N33Y; (b) the position 34 substitution comprises D34N; (c) the position 157 substitution comprises I157L; (d) the position 178 substitution comprises L178F; (e) the position 179 substitution comprises A179T. (f) the position 223 substitution comprises G223A; or (g) the position 307 substitution comprises H307L.

45 In highly preferred embodiments, the PS4 variant polypeptide comprises the substitutions: G134R, A141P, I157L, G223A, H307L and S334P, together with phenylalanine at position 178 or threonine at position 179, or both, optionally together with one or both of N33Y and D34N.

It may further comprise a substitution at position 121. The PS4 variant polypeptide
50 may have the sequence PSac-D34 (SEQ ID NO: 2) or the sequence PStu-D34 (SEQ ID NO: 8). The PS4 variant polypeptide may furthermore preferably be one in which the position 121 substitution comprises G121D. Preferably, the PS4 variant polypeptide has the sequence PSac-D20 (SEQ ID NO: 3) or the sequence PStu-D20 (SEQ ID NO: 9).

Further substitutions are possible. For example, the PS4 variant polypeptide may
55 further comprise a substitution at position 87. The position 87 substitution preferably comprises G87S. Preferably, the PS4 variant polypeptide has the sequence PSac-D14 (SEQ ID NO: 4) or the sequence PStu-D14 (SEQ ID NO: 10). The PS4 variant polypeptide may have the sequence PSac-pPD77d33.

According to a second aspect of the invention, we provide a use of a PS4 variant
60 polypeptide as set out in the first aspect of the invention as a food additive.

According to a third aspect of the invention, we provide a process for treating a starch comprising contacting the starch with a PS4 variant polypeptide as set out above and allowing the polypeptide to generate from the starch one or more linear products.

According to a fourth aspect of the invention, we provide use of a PS4 variant
65 polypeptide as set out in the first aspect of the invention in preparing a food product.

According to a fifth aspect of the invention, we provide a process of preparing a food product comprising admixing a polypeptide as set out in the first aspect of the invention with a food ingredient.

In preferred embodiments, the food product comprises a dough or a dough product,
70 preferably a processed dough product. Preferably, the food product is a bakery product.

According to a sixth aspect of the invention, we provide a process for making a bakery product comprising: (a) providing a starch medium; (b) adding to the starch medium a PS4 variant polypeptide as set out in the first aspect of the invention; and (c) applying heat to the starch medium during or after step (b) to produce a bakery product.

According to a seventh aspect of the invention, we provide a food product, dough
75 product or a bakery product obtained by a process as described.

According to a eighth aspect of the invention, we provide an improver composition for a dough, in which the improver composition comprises a PS4 variant polypeptide as set out in the first aspect of the invention, and at least one further dough ingredient or
80 dough additive.

According to a ninth aspect of the invention, we provide a composition comprising a flour and a PS4 variant polypeptide as set out in the first aspect of the invention.

According to a tenth aspect of the invention, we provide a use of a PS4 variant polypeptide as set out in the first aspect of the invention, in a dough product to retard or
85 reduce staling, preferably detrimental retrogradation, of the dough product.

According to a eleventh aspect of the invention, we provide a combination of a PS4 variant polypeptide as set out above, together with Novamyl, or a variant, homologue, or mutants thereof which has maltogenic alpha-amylase activity.

According to a twelfth aspect of the invention, we provide a use of a Novamyl
90 combination as described for an application as set out above.

According to an thirteenth aspect of the invention, we provide a food product
produced by treatment with a combination as described.

There is provided, according to a fourteenth aspect of the present invention, a food
additive comprising a PS4 variant polypeptide, in which the PS4 variant polypeptide is
95 derivable from a parent polypeptide having non-maltogenic exoamylase activity, in which
the PS4 variant polypeptide comprises substitutions at the following positions: G121D,
134, 141, 157, G223A, 307 and 334 with reference to the position numbering of a
Pseudomonas saccharophila exoamylase sequence shown as SEQ ID NO: 1.

BRIEF DESCRIPTION OF THE DRAWINGS

100 Figure 1 is a graph showing thermostability improvement of the PS4 variants.
PS4cc1 is an expressed control enzyme derived from *Pseudomonas saccharophila*,
without signal sequence and lacking the starch binding domain. Half life in minutes is
plotted against temperature in degrees C for PS4cc1, PSac-D3, PSac-D20 and PSac-D14.

Figure 2 is a graph showing dosage effect of PSac-D34 in a model baking system
105 trial. Solid content of crumb was measured by NMR. The effect on retrogradation
measured as solid content of the crumb is plotted against days after baking for control, 0.5,
1, 2 ppm of D34.

Figure 3 is a graph showing the results of a baking trial showing reduced firmness
and firming rate as upon adding PSac-D3 and Psac-D14 in a dosage of 1 mg per kg of
110 flour. Firmness measured by hPa is plotted against days after baking for control,

Figure 4 shows a baking trial showing the increased softening effect of PSac-D3
(N33Y, D34N, K71R, G134R, A141P, I157L, L178F, A179T, G223A, A307L, D343E,
S334P) compared to PSac-D3 without N33Y, D34N, K71R, L178F, A179T, which has
 $t_{1/2-75}$ of 3,6 in contrast to that of PSac-D3 being 9,3 min at 75C. Similar results are
115 obtained with a variant of PSac-D3 lacking the mutations at N33Y, D34N.

SEQUENCE LISTINGS

SEQ ID NO: 1 shows a PS4 reference sequence, derived from *Pseudomonas*
saccharophila maltotetrahydrolase amino acid sequence.

SEQ ID NO: 2 shows a PSac-D34 sequence; *Pseudomonas saccharophila*
120 maltotetrahydrolase amino acid sequence with 11 substitutions and deletion of the starch
binding domain.

SEQ ID NO: 3 shows a PSac-D20 sequence; *Pseudomonas saccharophila*
maltotetrahydrolase amino acid sequence with 13 substitutions and deletion of the starch
binding domain.

125 SEQ ID NO: 4 shows a PSac-D14 sequence; *Pseudomonas saccharophila*
maltotetrahydrolase amino acid sequence with 14 substitutions and deletion of the starch
binding domain.

SEQ ID NO: 5 shows a *Pseudomonas saccharophila* Glucan 1,4-alpha-
maltotetrahydrolase precursor (EC 3.2.1.60) (G4-amylase) (Maltotetraose-forming
130 amylase) (Exo-maltotetraohydrolase) (Maltotetraose-forming exo-amylase). SWISS-
PROT accession number P22963.

SEQ ID NO: 6 shows a *P. saccharophila* mta gene encoding maltotetraohydrolase
(EC number = 3.2.1.60). GenBank accession number X16732.

SEQ ID NO:7 shows a PS4 reference sequence, derived from *Pseudomonas*
135 *stutzeri* maltotetrahydrolase amino acid sequence.

SEQ ID NO: 8 shows a PStu-D34 sequence; *Pseudomonas stutzeri*
maltotetrahydrolase amino acid sequence with 9 substitutions.

SEQ ID NO: 9 shows a PStu-D20 sequence; *Pseudomonas stutzeri*
maltotetrahydrolase amino acid sequence with 11 substitutions.

140 SEQ ID NO: 10 shows a PStu-D14 sequence; *Pseudomonas stutzeri*
maltotetrahydrolase amino acid sequence with 12 substitutions.

SEQ ID NO: 11 shows a *Pseudomonas stutzeri* (*Pseudomonas perfectomarina*).
Glucan 1,4-alpha-maltotetrahydrolase precursor (EC 3.2.1.60) (G4-amylase)
(Maltotetraose-forming amylase) (Exo-maltotetraohydrolase)(Maltotetraose-forming exo-
145 amylase). SWISS-PROT accession number P13507.

SEQ ID NO: 12 shows a *P.stutzeri* maltotetraose-forming amylase (amyP) gene,
complete cds. GenBank accession number M24516.

SEQ ID NO: 13 shows a PSac-pPD77d33 sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 10 substitutions (N33Y, D34N, G134R,
150 A141P, I157L, L178F, A179T, G223A, H307L, S334P) and deletion of the starch binding domain.

SEQ ID NO: 14 shows a PSac-D34(Y33N) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 10 substitutions and deletion of the starch binding domain.

155 SEQ ID NO: 15 shows a PSac-D20(Y33N) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 12 substitutions and deletion of the starch binding domain.

SEQ ID NO: 16 shows a PSac-D14(Y33N) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 13 substitutions and deletion
160 of the starch binding domain.

SEQ ID NO: 17 shows a PStu-D34(Y33N) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 8 substitutions.

SEQ ID NO: 18 shows a PStu-D20(Y33N) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 10 substitutions.

165 SEQ ID NO: 19 shows a PStu-D14(Y33N) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 11 substitutions.

SEQ ID NO: 20 shows a PSac-pPD77d33(Y33N) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 9 substitutions (D34N, G134R, A141P, I157L, L178F, A179T, G223A, H307L, S334P) and deletion of the starch
170 binding domain.

SEQ ID NO: 21 shows a PSac-D34(N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 10 substitutions and deletion of the starch binding domain.

SEQ ID NO: 22 shows a PSac-D20(N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 12 substitutions and deletion of the starch
175 binding domain.

SEQ ID NO: 23 shows a PSac-D14(N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 13 substitutions and deletion of the starch binding domain.

180 SEQ ID NO: 24 shows a PStu-D34(N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 8 substitutions.

SEQ ID NO: 25 shows a PStu-D20(N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 10 substitutions.

185 SEQ ID NO: 26 shows a PStu-D14(N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 11 substitutions.

SEQ ID NO: 27 shows a PSac-pPD77d33(N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 9 substitutions (N33Y, G134R, A141P, I157L, L178F, A179T, G223A, H307L, S334P) and deletion of the starch binding domain.

190 SEQ ID NO: 28 shows a PSac-D34(Y33N-N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 9 substitutions and deletion of the starch binding domain.

195 SEQ ID NO: 29 shows a PSac-D20(Y33N-N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 11 substitutions and deletion of the starch binding domain.

SEQ ID NO: 30 shows a PSac-D14(Y33N-N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 12 substitutions and deletion of the starch binding domain.

200 SEQ ID NO: 31 shows a PStu-D34(Y33N-N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 7 substitutions.

SEQ ID NO: 32 shows a PStu-D20(Y33N-N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 9 substitutions.

SEQ ID NO: 33 shows a PStu-D14(Y33N-N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 10 substitutions.

205 SEQ ID NO: 34 shows a PSac-pPD77d33(Y33N-N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 8 substitutions (G134R, A141P, I157L, L178F, A179T, G223A, H307L, S334P) and deletion of the starch binding domain.

Other sequences are also shown in the sequence listings. Each of these other
210 sequences, as well as those SEQ ID NO: 1 to 34 may (with the exception of SEQ ID NO: 1, 5, 6, 7, 11 and 12) may be used as PS4 variant polypeptides according to the methods and compositions described here. Each of these sequences may be employed as parent sequences.

DETAILED DESCRIPTION

215 In the following description and examples, unless the context dictates otherwise, dosages of PS4 variant polypeptides are given in parts per million (micrograms per gram) of flour. For example, "1 D34" as used in Table 2 indicates 1 part per million of pSac-D34 based on weight per weight. Preferably, enzyme quantities or amounts are determined based on activity assays as equivalents of pure enzyme protein measured with bovine
220 serum albumin (BSA) as a standard, using the assay described in Bradford (1976, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254).

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant
225 DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John
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240 "*Immunocytochemistry: Theory and Practice*", CRC Press inc., Boca Raton, Florida, 1988, ISBN 0-8493-6078-1, John D. Pound (ed); "*Immunochemical Protocols, vol 80*", in the series: "Methods in Molecular Biology", Humana Press, Totowa, New Jersey, 1998, ISBN 0-89603-493-3, Handbook of Drug Screening, edited by Ramakrishna Seethala, Prabhavathi B. Fernandes (2001, New York, NY, Marcel Dekker, ISBN 0-8247-0562-9);
245 and Lab Ref: A Handbook of Recipes, Reagents, and Other Reference Tools for Use at the Bench, Edited Jane Roskams and Linda Rodgers, 2002, Cold Spring Harbor Laboratory, ISBN 0-87969-630-3. Each of these general texts is herein incorporated by reference.

PS4 VARIANTS

We provide for compositions comprising polypeptides which are variants of
250 polypeptides having non-maltogenic exoamylase activity, as well as uses of such variant polypeptides and the compositions. The compositions include the polypeptide variants together with another component. In particular, we provide for food additives comprising the polypeptides.

Such variant polypeptides are referred to in this document as "PS4 variant
255 polypeptides". Nucleic acids encoding such variant polypeptides will be referred to for convenience as "PS4 variant nucleic acids". PS4 variant polypeptides and nucleic acids will be described in further detail below.

Specifically, we provide for PS4 variant polypeptides with sequence alterations comprising amino acid substitutions in a non-maltogenic exoamylase sequence. The amino
260 acid substitutions may be at any one or more of positions 134, 141, 157, 223, 307 and 334, with reference to the position numbering of a *Pseudomonas saccharophilia* exoamylase sequence shown as SEQ ID NO: 1. Further substitutions at one or both of positions 33 and 34 may be present. Even further substitutions at positions 178 and 179 may also be included.

265 The residue at position 33 is preferably wild type, i.e., N; similarly, the residue at position 34 is preferably wild type, i.e., D.

Where a substitution at position 121 is present, it will advantageously comprise G121D. Similarly, where a substitution at position 223 is present, it will advantageously comprise G223A. Alternatively, it may comprise G223L. Accordingly, in some
270 embodiments, we disclose a food additive comprising a PS4 variant polypeptide, in which the PS4 variant polypeptide is derivable from a parent polypeptide having non-maltogenic exoamylase activity, in which the PS4 variant polypeptide comprises substitutions at the following positions: G121D, 134, 141, 157, G223A, 307 and 334 with reference to the position numbering of a *Pseudomonas saccharophilia* exoamylase sequence shown as
275 SEQ ID NO: 1.

Examples of PS4 variant polypeptides are set out in the Sequence Listings, and they are described in detail below.

Such variant polypeptides retain the features of the parent polypeptides, and additionally preferably have additional beneficial properties, for example, enhanced
280 activity or thermostability, or pH resistance, or any combination (preferably all).

The PS4 substitution mutants described here may be used for any purpose for which the parent enzyme is suitable. In particular, they may be used in any application for which exo-maltotetrahydrolase is used. In highly preferred embodiments, they have the added advantage of higher thermostability, or higher exoamylase activity or higher pH
285 stability, or any combination. Examples of suitable uses for the PS4 variant polypeptides and nucleic acids include food production, in particular baking, as well as production of foodstuffs; further examples are set out in detail below.

The "parent" sequences, i.e., the sequences on which the PS4 variant polypeptides and nucleic acids are based, preferably are polypeptides having non-maltogenic
290 exoamylase activity. The terms "parent enzymes" and "parent polypeptides" should be interpreted accordingly, and taken to mean the enzymes and polypeptides on which the PS4 variant polypeptides are based.

In particularly preferred embodiments, the parent sequences are non-maltogenic exoamylase enzymes, preferably bacterial non-maltogenic exoamylase enzymes. In highly
295 preferred embodiments, the parent sequence comprises a glucan 1,4-alpha-maltotetrahydrolase (EC 3.2.1.60). Preferably, the parent sequence is from *Pseudomonas* species, for example *Pseudomonas saccharophilia* or *Pseudomonas stutzeri*.

In preferred embodiments, the parent polypeptide comprises, or is homologous to, a *Pseudomonas saccharophilia* non-maltogenic exoamylase having a sequence shown as
300 SEQ ID NO: 1. Proteins and nucleic acids related to, preferably having sequence or functional homology with *Pseudomonas saccharophilia* non-maltogenic exoamylase *Pseudomonas saccharophilia* exoamylase sequence shown as SEQ ID NO: 1 are referred to in this document as members of the "PS4 family". Examples of "PS4 family" non-maltogenic exoamylase enzymes suitable for use in generating the PS4 variant
305 polypeptides and nucleic acids are disclosed in further detail below.

In some preferred embodiments, the parent polypeptide comprises a non-maltogenic exoamylase from *Pseudomonas saccharophilia* non-maltogenic exoamylase having a sequence shown as SEQ ID NO: 1, or a SWISS-PROT accession number P22963. In other preferred embodiments, the parent polypeptide comprises a non-
310 maltogenic exoamylase from *Pseudomonas stutzeri* having a sequence shown as SEQ ID NO: 11, or a *Pseudomonas stutzeri* non-maltogenic exoamylase having SWISS-PROT accession number P13507.

The PS4 variant polypeptides and nucleic acids vary from their parent sequences by including a number of mutations. In other words, the sequence of the PS4 variant
315 polypeptide or nucleic acid is different from that of its parent at a number of positions or residues. In preferred embodiments, the mutations comprise amino acid substitutions, that is, a change of one amino acid residue for another. Thus, the PS4 variant polypeptides comprise a number of changes in the nature of the amino acid residue at one or more positions of the parent sequence.

320 In describing the different PS4 variant polypeptide variants produced or which are contemplated to be encompassed by this document, the following nomenclature will be adopted for ease of reference: [original amino acid/position according to the numbering system/substituted amino acid]. Accordingly, for example, the substitution of alanine with proline in position 141 is designated as A141P. Multiple mutations may be designated by
325 being separated by slash marks "/", e.g. A141P/G223A representing mutations in position 141 and 223 substituting alanine with proline and glycine with alanine respectively.

All positions referred to in the present document by numbering refer to the numbering of a *Pseudomonas saccharophilia* exoamylase reference sequence shown below (SEQ ID NO: 1):

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330   1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APNDWYNILR QQASTIAADG FSAIWMPVPW
      61 RDFSSWTDGG KSGGGEYFW HDFNKNRGYR SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
      121 GYPDKKEINLP AGQGFWRNDC ADPGNYPNDC DDGDRFIGGE SDLNTGHPQI YGMFRDELAN
      181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKGPSEYPSW DWRNTASWQQ
      241 IIKDWSRAK CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
335  301 QNGGQHHWAL QDGLIRQAYA YILTSPGTPV VYWSHMYDWG YGDFIRQLIQ VRRTAGVRAD
      361 SAISFHSYGS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
      421 GDGGGNDGGE GGLVNVNFRD DNGVTQMGS VYAVGNVSQL GNWSPASAVR LTDTSSSYPTW
      481 KGSIALPDGQ NVEWKCLIRN EADATLVRQW QSGGNNQVQA AAGASTSGSF

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The reference sequence is derived from the *Pseudomonas saccharophilia* sequence
 340 having SWISS-PROT accession number P22963, but without the signal sequence
 MSHILRAAVLAAVLLPFPALA.

The PS4 variant polypeptide variants described here preferably comprises
 substitutions at the following positions: G134, A141, I157, G223, H307, S334, N33 and
 D34. Preferably, they may comprise in addition substitutions at one or both of L178 and
 345 A179. In further preferred embodiments, glycine at position 134 is substituted by arginine
 in the PS4 variant polypeptides. In further particularly preferred embodiments, arginine at
 position 141 is substituted by proline. Furthermore, in such particularly preferred
 embodiments, serine at position 334 is substituted by proline.

Accordingly, in preferred embodiments, the PS4 variant polypeptides comprise
 350 substitutions arginine at position 134, proline at position 141 and proline at position 334,
 e.g., G134R, A141P and S334P. The residues at positions 157, 223, 307, 33, 34, 178 and
 179, may be substituted by a number of residues, for example I157V or I157N or G223L
 or G223I or G223S or G223T or H307I or H307V or D34G or D34A or D34S or D34T or
 A179V.

355 However, the PS4 variant polypeptides preferably comprise the substitutions
 I157L, L178F, A179T G223A and H307L. Where substitutions at positions 33 and/or 34
 are present, these will preferably be N33Y and D34N.

In highly preferred embodiments, the PS4 variant polypeptides comprise the
 following substitutions: G134R, A141P, I157L, G223A, H307L and S334P, together with
 360 one or both of L178F and A179T. Optionally, substitutions N33Y and D34N may also be
 included.

In one embodiment, the PS4 variants are derived from a *Pseudomonas saccharophila* non-maltogenic enzyme sequence. Accordingly, and preferably, the PS4 variant polypeptide variant comprises a sequence PSac-D34 (SEQ ID NO: 2).

365 One or more further substitutions may be introduced into the parent sequence, in particular, at G121 or G87, or at both positions. The G121 substitution preferably comprises G121D, and the G87 substitution preferably comprises G87S.

Accordingly, we disclose *Pseudomonas saccharophila* based PS4 variant polypeptides comprising the following substitutions: G134R, A141P, I157L, G121D, 370 G223A, H307L and S334P, together with one or both of L178F and A179T, optionally also N33Y and/or D34N, as well as PS4 variant polypeptides comprising the following substitutions: G87S, G121D, G134R, A141P, I157L, G223A, H307L and S334P, together with one or both of L178F and A179T, optionally together with N33Y and/or D34N.

Therefore, a PS4 variant based on *Pseudomonas saccharophila* non-maltogenic 375 enzyme sequence may have a sequence PSac-D20 (SEQ ID NO: 3), comprising G121D, or a sequence PSac-D14 (SEQ ID NO: 4), further comprising G87S. The PS4 variant polypeptide may have the sequence PSac-pPD77d33.

In another embodiment, the PS4 variants are derived from a *Pseudomonas stutzeri* non-maltogenic enzyme sequence, preferably shown as SEQ ID NO: 7 below:

380 1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APNDWYNILR QQAATIAADG FSAIWMPPVPW
61' RDFSSWSDGS KSGGGEYFW HDFNKNRGYR SDAQLRQAAS ALGGAGVKVL YDVPNHNMR
121 GYPDKENLIP AGQGFWRNDC ADPGNYPNDC DDGDRFIGGD ADLNTGHPQV YGMFRDEFNT
181 LRSQYGAGGF RFDFVRGYAP ERVNSWMTDS ADNSFCVGEL WKGPSSEYPNW DWRNTASWQQ
241 IIKDWSDRAK CPVFDFALKE RMQNGSIADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
385 301 QNGGQHHWAL QDGLIRQAYA YILTSPGTPV VYWSHMYDWG YGDFIRQLIQ VRRAGVRAD
361 SAISFHSYGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSQT
421 GSGGGEPPGAL VSVSFRCDNG ATQMGSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

Accordingly, the PS4 variant polypeptide may comprise a sequence PStu-D34 390 (SEQ ID NO: 8). We further disclose PS4 variant polypeptides based on *Pseudomonas stutzeri* non-maltogenic enzyme sequence and including G121 and / or G87 substitutions. These may comprise the following substitutions: N33Y (if a substitution at position 33 is present), D34N (if a substitution at position 34 is present), G121D, G134R, A141P, I157L, G223A, H307L and S334P, together with one or both of L178F and A179T, as well as 395 PS4 variant polypeptides comprising the following substitutions: N33Y (if a substitution at position 33 is present), D34N (if a substitution at position 34 is present), G87S, G121D,

G134R, A141P, I157L, G223A, H307L and S334P, together with one or both of L178F and A179T.

400 A PS4 variant based on *Pseudomonas stutzeri* non-maltogenic enzyme sequence may have a sequence PStu-D20 (SEQ ID NO: 9), comprising G121D, or a sequence PStu-D14 (SEQ ID NO: 10), further comprising G87S.

405 The numbering system, even though it may use a specific sequence as a base reference point, is also applicable to all relevant homologous sequences. For example, the position numbering may be applied to homologous sequences from other *Pseudomonas* species, or homologous sequences from other bacteria. Preferably, such homologous have 60% or greater homology, for example 70% or more, 80% or more, 90% or more or 95% or more homology, with the reference sequence SEQ ID NO: 1 above, or the sequences having SWISS-PROT accession numbers P22963 or P13507, preferably with all these sequences. Sequence homology between proteins may be ascertained using well known alignment programs and hybridisation techniques described herein. Such homologous 410 sequences will be referred to in this document as the "PS4 Family".

Furthermore, and as noted above, the numbering system used in this document makes reference to a reference sequence SEQ ID NO: 1, which is derived from the *Pseudomonas saccharophilia* sequence having SWISS-PROT accession number P22963, 415 but without the signal sequence MSHILRAAVLA VLLPFPALA. This signal sequence is located N terminal of the reference sequence and consists of 21 amino acid residues. Accordingly, it will be trivial to identify the particular residues to be mutated or substituted in corresponding sequences comprising the signal sequence, or indeed, corresponding sequences comprising any other N- or C- terminal extensions or deletions. 420 For example, the sequence of *Pseudomonas saccharophilia* non-maltogenic exoamylase having SWISS-PROT accession number P22963 or a *Pseudomonas stutzeri* non-maltogenic exoamylase having SWISS-PROT accession number P13507.

425 The PS4 variant polypeptides may comprise one or more further mutations in addition to those set out above. There may be one, two, three, four, five, six, seven or more mutations preferably substitutions in addition to those already set out. Other mutations, such as deletions, insertions, substitutions, transversions, transitions and inversions, at one or more other locations, may also be included. In addition, the PS4

variants need not have all the substitutions at the positions listed. Indeed, they may have one, two, three, four, or five substitutions missing, i.e., the wild type amino acid residue is
430 present at such positions.

We also describe PS4 nucleic acids having sequences which correspond to or encode the alterations in the PS4 variant polypeptide sequences, for use in producing such polypeptides for the purposes described here. The skilled person will be aware of the relationship between nucleic acid sequence and polypeptide sequence, in particular, the
435 genetic code and the degeneracy of this code, and will be able to construct such PS4 nucleic acids without difficulty. For example, he will be aware that for each amino acid substitution in the PS4 variant polypeptide sequence, there may be one or more codons which encode the substitute amino acid. Accordingly, it will be evident that, depending on the degeneracy of the genetic code with respect to that particular amino acid residue, one
440 or more PS4 nucleic acid sequences may be generated corresponding to that PS4 variant polypeptide sequence. Furthermore, where the PS4 variant polypeptide comprises more than one substitution, for example A141P/G223A, the corresponding PS4 nucleic acids may comprise pairwise combinations of the codons which encode respectively the two amino acid changes.

445 Thus, for example, a PS4 nucleic acid sequence may be derivable from a parent sequence encoding a polypeptide having non-maltogenic exoamylase activity and comprising codons encoding amino acid substitutions at the following positions: G134, A141, I157, G223, H307, S334, optionally with one or both of N33 and D34, together with one or both of L178 and A179, with reference to the position numbering of a
450 *Pseudomonas saccharophilia* exoamylase sequence shown as SEQ ID NO: 1. We also describe a nucleic acid sequence derivable from a parent sequence, the parent sequence capable of encoding a non-maltogenic exoamylase, which nucleic acid sequence comprises a substitution at one or more residues such that the nucleic acid encodes one or more of the following mutations at the positions specified: G134, A141, I157, G223,
455 H307, S334, optionally together with one or both of N33 and D34, together with one or both of L178 and A179, with reference to the position numbering of a *Pseudomonas saccharophilia* exoamylase sequence shown as SEQ ID NO: 1.

It will be understood that nucleic acid sequences which are not identical to the particular PS4 variant nucleic acid sequences, but are related to these, will also be useful for the methods and compositions described here, such as a variant, homologue, derivative or fragment of a PS4 variant nucleic acid sequence, or a complement or a sequence capable of hybridising thereof. Unless the context dictates otherwise, the term "PS4 variant nucleic acid" should be taken to include each of these entities listed above.

Mutations in amino acid sequence and nucleic acid sequence may be made by any of a number of techniques, as known in the art. In particularly preferred embodiments, the mutations are introduced into parent sequences by means of PCR (polymerase chain reaction) using appropriate primers, as illustrated in the Examples. It is therefore possible to alter the sequence of a polypeptide by introducing amino acid substitutions comprising: G134, A141, I157, G223, H307, S334, optionally N33 and/or D34, together with one or both of L178 and A179, into a parent polypeptide having non-maltogenic exoamylase activity, such as into a *Pseudomonas saccharophilia* or a *Pseudomonas stutzeri* exoamylase sequence at amino acid or nucleic acid level, as described. We describe a method in which the sequence of a non-maltogenic exoamylase is altered by altering the sequence of a nucleic acid which encodes the non-maltogenic exoamylase.

The PS4 variant polypeptides and nucleic acids may be produced by any means known in the art. Specifically, they may be expressed from expression systems, which may be *in vitro* or *in vivo* in nature. Specifically, we describe plasmids and expression vectors comprising PS4 nucleic acid sequences, preferably capable of expressing PS4 variant polypeptides. Cells and host cells which comprise and are preferably transformed with such PS4 nucleic acids, plasmids and vectors are also disclosed, and it should be made clear that these are also encompassed in this document.

In preferred embodiments, the PS4 variant polypeptide sequence is used as a food additive in an isolated form. The term "isolated" means that the sequence is at least substantially free from at least one other component with which the sequence is naturally associated in nature and as found in nature. In one aspect, preferably the sequence is in a purified form. The term "purified" means that the sequence is in a relatively pure state — e.g. at least about 90% pure, or at least about 95% pure or at least about 98% pure.

PARENT ENZYME

The PS4 variant polypeptides are derived from, or are variants of, another
490 sequence, known as a "parent enzyme", a "parent polypeptide" or a "parent sequence".

The term "parent enzyme" as used in this document means the enzyme that has a close, preferably the closest, chemical structure to the resultant variant, i.e., the PS4 variant polypeptide or nucleic acid. The parent enzyme may be a precursor enzyme (i.e. the enzyme that is actually mutated) or it may be prepared *de novo*. The parent enzyme
495 may be a wild type enzyme.

The term "precursor" as used herein means an enzyme that precedes the enzyme which is modified to produce the enzyme. Thus, the precursor may be an enzyme that is modified by mutagenesis. Likewise, the precursor may be a wild type enzyme, a variant wild type enzyme or an already mutated enzyme.

500 The term "wild type" is a term of the art understood by skilled persons and means a phenotype that is characteristic of most of the members of a species occurring naturally and contrasting with the phenotype of a mutant. Thus, in the present context, the wild type enzyme is a form of the enzyme naturally found in most members of the relevant species. Generally, the relevant wild type enzyme in relation to the variant polypeptides described
505 here is the most closely related corresponding wild type enzyme in terms of sequence homology. However, where a particular wild type sequence has been used as the basis for producing a variant PS4 polypeptide as described here, this will be the corresponding wild type sequence regardless of the existence of another wild type sequence that is more closely related in terms of amino acid sequence homology.

510 The parent enzyme is preferably a polypeptide which preferably exhibits non-maltogenic exoamylase activity. Preferably, the parent enzyme is a non-maltogenic exoamylase itself. For example, the parent enzyme may be a *Pseudomonas saccharophila* non-maltogenic exoamylase, such as a polypeptide having SWISS-PROT accession number P22963, or a *Pseudomonas stutzeri* non-maltogenic exoamylase, such as a
515 polypeptide having SWISS-PROT accession number P13507. Other members of the PS4 family may be used as parent enzymes; such PS4 family members will generally be similar to, homologous to, or functionally equivalent to either of these two enzymes, and

may be identified by standard methods, such as hybridisation screening of a suitable library using probes, or by genome sequence analysis.

520 In particular, functional equivalents of either of these two enzymes, as well as other members of the "PS4 family" may also be used as starting points or parent polypeptides for the generation of PS4 variant polypeptides as described here.

A "functional equivalent" of a protein means something that shares one or more, preferably substantially all, of the functions of that protein. Preferably, such functions are
525 biological functions, preferably enzymatic functions, such as amylase activity, preferably non-maltogenic exoamylase activity.

The term "functional equivalent" in relation to a parent enzyme being a *Pseudomonas saccharophila* non-maltogenic exoamylase, such as a polypeptide having SWISS-PROT accession number P22963, or a *Pseudomonas stutzeri* non-maltogenic
530 exoamylase, such as a polypeptide having SWISS-PROT accession number P13507 means that the functional equivalent could be obtained from other sources. The functionally equivalent enzyme may have a different amino acid sequence but will have non-maltogenic exoamylase activity.

In highly preferred embodiments, the functional equivalent will have sequence
535 homology to either of the *Pseudomonas saccharophila* and *Pseudomonas stutzeri* non-maltogenic exoamylases mentioned above, preferably both. The functional equivalent may also have sequence homology with any of the sequences set out as SEQ ID NOs: 1 to 12, preferably SEQ ID NO: 1 or SEQ ID NO: 7 or both. Sequence homology between such sequences is preferably at least 60%, preferably 65% or more, preferably 75% or more,
540 preferably 80% or more, preferably 85% or more, preferably 90% or more, preferably 95% or more. Such sequence homologies may be generated by any of a number of computer programs known in the art, for example BLAST or FASTA, etc. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A; Devereux *et al.*, 1984, Nucleic Acids Research
545 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410) and the GENWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching

(see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG
550 Bestfit program.

In other embodiments, the functional equivalents will be capable of specifically hybridising to any of the sequences set out above. Methods of determining whether one sequence is capable of hybridising to another are known in the art, and are for example described in Sambrook, et al (*supra*) and Ausubel, F. M. et al. (*supra*). In highly preferred
555 embodiments, the functional equivalents will be capable of hybridising under stringent conditions, e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na₃ Citrate pH 7.0}.

For example, functional equivalents which have sequence homology to *Pseudomonas saccharophila* and *Pseudomonas stutzeri* non-maltogenic exoamylases are suitable for use as parent enzymes. Such sequences may differ from the *Pseudomonas*
560 *saccharophila* sequence at any one or more positions. Furthermore, non-maltogenic exoamylases from other strains of *Pseudomonas* spp, such as ATCC17686, may also be used as a parent polypeptide. The PS4 variant polypeptide residues may be inserted into any of these parent sequences to generate the variant PS4 polypeptide sequences.

The parent enzymes may be modified at the amino acid level or the nucleic acid
565 level to generate the PS4 variant sequences described here. Therefore, we provide for the generation of PS4 variant polypeptides by introducing one or more corresponding codon changes in the nucleotide sequence encoding a non-maltogenic exoamylase polypeptide.

The nucleic acid numbering should preferably be with reference to the position numbering of a *Pseudomonas saccharophila* exoamylase nucleotide sequence shown as
570 SEQ ID NO: 6. Alternatively, or in addition, reference may be made to the sequence with GenBank accession number X16732. In preferred embodiments, the nucleic acid numbering should be with reference to the nucleotide sequence shown as SEQ ID NO: 6. However, as with amino acid residue numbering, the residue numbering of this sequence is to be used only for reference purposes only. In particular, it will be appreciated that the
575 above codon changes can be made in any PS4 family nucleic acid sequence. For example, sequence changes can be made to a *Pseudomonas saccharophila* or a *Pseudomonas stutzeri* non-maltogenic exoamylase nucleic acid sequence (e.g., X16732, SEQ ID NO: 6 or M24516, SEQ ID NO: 12).

The parent enzyme may comprise the “complete” enzyme, i.e., in its entire length
580 as it occurs in nature (or as mutated), or it may comprise a truncated form thereof. The
PS4 variant derived from such may accordingly be so truncated, or be “full-length”. The
truncation may be at the N-terminal end, or the C-terminal end, preferably the C-terminal
end. The parent enzyme or PS4 variant may lack one or more portions, such as sub-
sequences, signal sequences, domains or moieties, whether active or not etc. For example,
585 the parent enzyme or the PS4 variant polypeptide may lack a signal sequence, as described
above. Alternatively, or in addition, the parent enzyme or the PS4 variant may lack one or
more catalytic or binding domains.

In highly preferred embodiments, the parent enzyme or PS4 variant may lack one
or more of the domains present in non-maltogenic exoamylases, such as the starch binding
590 domain. For example, the PS4 polypeptides may have only sequence up to position 429,
relative to the numbering of a *Pseudomonas saccharophilia* non-maltogenic exoamylase
shown as SEQ ID NO: 1. It is to be noted that this is the case for the PS4 variants pSac-
d34, pSac-D20 and pSac-D14.

AMYLASE

595 The PS4 variant polypeptides generally comprise amylase activity.

The term “amylase” is used in its normal sense - e.g. an enzyme that is *inter alia*
capable of catalysing the degradation of starch. In particular they are hydrolases which are
capable of cleaving α -D-(1 \rightarrow 4) O-glycosidic linkages in starch.

Amylases are starch-degrading enzymes, classified as hydrolases, which cleave α -
600 D-(1 \rightarrow 4) O-glycosidic linkages in starch. Generally, α -amylases (E.C. 3.2.1.1, α -D-
(1 \rightarrow 4)-glucan glucanohydrolase) are defined as endo-acting enzymes cleaving α -D-(1 \rightarrow 4)
O-glycosidic linkages within the starch molecule in a random fashion. In contrast, the exo-
acting amylolytic enzymes, such as β -amylases (E.C. 3.2.1.2, α -D-(1 \rightarrow 4)-glucan
maltohydrolase), and some product-specific amylases like maltogenic alpha-amylase (E.C.
605 3.2.1.133) cleave the starch molecule from the non-reducing end of the substrate. β -
Amylases, α -glucosidases (E.C. 3.2.1.20, α -D-glucoside glucohydrolase), glucoamylase
(E.C. 3.2.1.3, α -D-(1 \rightarrow 4)-glucan glucohydrolase), and product-specific amylases can
produce malto-oligosaccharides of a specific length from starch.

NON-MALTOGENIC EXOAMYLASE

610 The PS4 variant polypeptides described in this document are derived from (or variants of) polypeptides which preferably exhibit non-maltogenic exoamylase activity. Preferably, these parent enzymes are non-maltogenic exoamylases themselves. The PS4 variant polypeptides themselves in highly preferred embodiments also exhibit non-maltogenic exoamylase activity.

615 In highly preferred embodiments, the term "non-maltogenic exoamylase enzyme" as used in this document should be taken to mean that the enzyme does not initially degrade starch to substantial amounts of maltose as analysed in accordance with the product determination procedure as described in this document.

In highly preferred embodiments, the non-maltogenic exoamylase comprises an
620 exo-maltotetrahydrolase. Exo-maltotetrahydrolase (E.C.3.2.1.60) is more formally known as glucan 1,4-alpha-maltotetrahydrolase. This enzyme hydrolyses 1,4-alpha-D-glucosidic linkages in amylaceous polysaccharides so as to remove successive maltotetraose residues from the non-reducing chain ends.

Non-maltogenic exoamylases and their uses are described in detail in US Patent
625 number 6,667,065, as well as WO99/50399 hereby incorporated by reference.

ASSAYS FOR NON-MALTOGENIC EXOAMYLASE ACTIVITY

The following system is used to characterize polypeptides having non-maltogenic exoamylase activity which are suitable for use according to the methods and compositions described here. This system may for example be used to characterise the PS4 parent or
630 variant polypeptides described here.

By way of initial background information, waxy maize amylopectin (obtainable as WAXILYS 200 from Roquette, France) is a starch with a very high amylopectin content (above 90%). 20 mg/ml of waxy maize starch is boiled for 3 min. in a buffer of 50 mM MES (2-(N-morpholino)ethanesulfonic acid), 2 mM calcium chloride, pH 6.0 and
635 subsequently incubated at 50°C and used within half an hour.

One unit of the non-maltogenic exoamylase is defined as the amount of enzyme which releases hydrolysis products equivalent to 1 μ mol of reducing sugar per min. when incubated at 50 degrees C in a test tube with 4 ml of 10 mg/ml waxy maize starch in 50

mM MES, 2 mM calcium chloride, pH 6.0 prepared as described above. Reducing sugars
640 are measured using maltose as standard and using the dinitrosalicylic acid method of
Bernfeld, *Methods Enzymol.*, (1954), 1, 149-158 or another method known in the art for
quantifying reducing sugars.

The hydrolysis product pattern of the non-maltogenic exoamylase is determined by
incubating 0.7 units of non-maltogenic exoamylase for 15 or 300 min. at 50°C in a test
645 tube with 4 ml of 10 mg/ml waxy maize starch in the buffer prepared as described above.
The reaction is stopped by immersing the test tube for 3 min. in a boiling water bath.

The hydrolysis products are analyzed and quantified by anion exchange HPLC
using a Dionex PA 100 column with sodium acetate, sodium hydroxide and water as
eluent, with pulsed amperometric detection and with known linear maltooligosaccharides
650 of from glucose to maltoheptaose as standards. The response factor used for maltooctaose
to maltodecaose is the response factor found for maltoheptaose.

Preferably, the PS4 variant polypeptides have non-maltogenic exoamylase activity
such that if an amount of 0.7 units of said non-maltogenic exoamylase were to incubated
for 15 minutes at a temperature of 50°C at pH 6.0 in 4 ml of an aqueous solution of 10 mg
655 preboiled waxy maize starch per ml buffered solution containing 50 mM 2-(N-
morpholino)ethane sulfonic acid and 2 mM calcium chloride then the enzyme would yield
hydrolysis product(s) that would consist of one or more linear malto-oligosaccharides of
from two to ten D-glucopyranosyl units and optionally glucose; such that at least 60%,
preferably at least 70%, more preferably at least 80% and most preferably at least 85% by
660 weight of the said hydrolysis products would consist of linear maltooligosaccharides of
from three to ten D-glucopyranosyl units, preferably of linear maltooligosaccharides
consisting of from four to eight D-glucopyranosyl units.

For ease of reference, and for the present purposes, the feature of incubating an
amount of 0.7 units of the non-maltogenic exoamylase for 15 minutes at a temperature of
665 50°C at pH 6.0 in 4 ml of an aqueous solution of 10 mg preboiled waxy maize starch per
ml buffered solution containing 50 mM 2-(N-morpholino)ethane sulfonic acid and 2 mM
calcium chloride, may be referred to as the "Waxy Maize Starch Incubation Test".

Thus, alternatively expressed, preferred PS4 variant polypeptides which are non-
maltogenic exoamylases are characterised as having the ability in the waxy maize starch

670 incubation test to yield hydrolysis product(s) that would consist of one or more linear
malto-oligosaccharides of from two to ten D-glucopyranosyl units and optionally glucose;
such that at least 60%, preferably at least 70%, more preferably at least 80% and most
preferably at least 85% by weight of the said hydrolysis product(s) would consist of linear
maltooligosaccharides of from three to ten D-glucopyranosyl units, preferably of linear
675 maltooligosaccharides consisting of from four to eight D-glucopyranosyl units.

The hydrolysis products in the waxy maize starch incubation test may include one
or more linear malto-oligosaccharides of from two to ten D-glucopyranosyl units and
optionally glucose. The hydrolysis products in the waxy maize starch incubation test may
also include other hydrolytic products. Nevertheless, the % weight amounts of linear
680 maltooligosaccharides of from three to ten D-glucopyranosyl units are based on the
amount of the hydrolysis product that consists of one or more linear malto-
oligosaccharides of from two to ten D-glucopyranosyl units and optionally glucose. In
other words, the % weight amounts of linear maltooligosaccharides of from three to ten D-
glucopyranosyl units are not based on the amount of hydrolysis products other than one or
685 more linear malto-oligosaccharides of from two to ten D-glucopyranosyl units and
glucose.

The hydrolysis products can be analysed by any suitable means. For example, the
hydrolysis products may be analysed by anion exchange HPLC using a Dionex PA 100
column with pulsed amperometric detection and with, for example, known linear
690 maltooligosaccharides of from glucose to maltoheptaose as standards.

For ease of reference, and for the present purposes, the feature of analysing the
hydrolysis product(s) using anion exchange HPLC using a Dionex PA 100 column with
pulsed amperometric detection and with known linear maltooligosaccharides of from
glucose to maltoheptaose used as standards, can be referred to as "analysing by anion
695 exchange". Of course, and as just indicated, other analytical techniques would suffice, as
well as other specific anion exchange techniques.

Thus, alternatively expressed, a preferred PS4 variant polypeptide is one which has
non-maltogenic exoamylase such that it has the ability in a waxy maize starch incubation
test to yield hydrolysis product(s) that would consist of one or more linear malto-
700 oligosaccharides of from two to ten D-glucopyranosyl units and optionally glucose, said

hydrolysis products being capable of being analysed by anion exchange; such that at least 60%, preferably at least 70%, more preferably at least 80% and most preferably at least 85% by weight of the said hydrolysis product(s) would consist of linear maltooligosaccharides of from three to ten D-glucopyranosyl units, preferably of linear
705 maltooligosaccharides consisting of from four to eight D-glucopyranosyl units.

As used herein, the term "linear malto-oligosaccharide" is used in the normal sense as meaning 2-10 units of α -D-glucopyranose linked by an α -(1 \rightarrow 4) bond.

In highly preferred embodiments, the PS4 polypeptides described here have improved exoamylase activity, preferably non-maltogenic exoamylase activity, when
710 compared to the parent polypeptide, preferably when tested under the same conditions. In particular, in highly preferred embodiments, the PS4 variant polypeptides have 10% or more, preferably 20% or more, preferably 50% or more, exoamylase activity compared to their parents, preferably when measured in a waxy maize starch test.

The hydrolysis products can be analysed by any suitable means. For example, the
715 hydrolysis products may be analysed by anion exchange HPLC using a Dionex PA 100 column with pulsed amperometric detection and with, for example, known linear maltooligosaccharides of from glucose to maltoheptaose as standards.

As used herein, the term "linear malto-oligosaccharide" is used in the normal sense as meaning 2-20 units of α -D-glucopyranose linked by an α -(1 \rightarrow 4) bond.

720 IMPROVED PROPERTIES

The PS4 variants described here preferably have improved properties when compared to their parent enzymes, such as any one or more of improved thermostability, improved pH stability, or improved exo-specificity.

Without wishing to be bound by any particular theory, we believe that the
725 mutations at the particular positions have individual and cumulative effects on the properties of a polypeptide comprising such mutations. Thus, for example, we believe that positions 134, 141, 157, 223, 334, as well as optionally positions 178 or 179, or both influence the thermostability of PS4 polypeptides comprising such changes. Particularly, and preferably, positive or beneficial effects reside in these positions, particular in the
730 substitutions: 134R, 141P, 157L, 223A, 307L, 334P, 178F and 179T where present.

On the other hand, we believe that positions 307, as well as position 121 have effects (preferably positive effects) on the exo-specificity of a PS4 polypeptide.

THERMOSTABILITY AND PH STABILITY

Preferably, the PS4 variant polypeptide is thermostable; preferably, it has higher
735 thermostability than its parent enzyme.

In wheat and other cereals the external side chains in amylopectin are in the range of DP 12-19. Thus, enzymatic hydrolysis of the amylopectin side chains, for example, by PS4 variant polypeptides as described having non-maltogenic exoamylase activity, can markedly reduce their crystallisation tendencies.

740 Starch in wheat and other cereals used for baking purposes is present in the form of starch granules which generally are resistant to enzymatic attack by amylases. Thus starch modification is mainly limited to damaged starch and is progressing very slowly during dough processing and initial baking until gelatinisation starts at about 60C. As a consequence hereof only amylases with a high degree of thermostability are able to
745 modify starch efficiently during baking. And generally the efficiency of amylases is increased with increasing thermostability. That is because the more thermostable the enzyme is the longer time it can be active during baking and thus the more antistaling effect it will provide.

Accordingly, the use of PS4 variant polypeptides as described here when added to
750 the starch at any stage of its processing into a food product, e.g., before during or after baking into bread can retard or impede or slow down the retrogradation. Such use is described in further detail below.

As used herein the term 'thermostable' relates to the ability of the enzyme to retain activity after exposure to elevated temperatures. Preferably, the PS4 variant polypeptide is
755 capable of degrading starch at temperatures of from about 55°C to about 80°C or more. Suitably, the enzyme retains its activity after exposure to temperatures of up to about 95°C.

The thermostability of an enzyme such as a non-maltogenic exoamylase is measured by its half life. Thus, the PS4 variant polypeptides described here have half lives
760 extended relative to the parent enzyme by preferably 10%, 20%, 30%, 40%, 50%, 60%,

70%, 80%, 90%, 100%, 200% or more, preferably at elevated temperatures of from 55°C to about 95°C or more, preferably at about 80°C.

As used here, the half life ($t_{1/2}$) is the time (in minutes) during which half the enzyme activity is inactivated under defined heat conditions. In preferred embodiments, the half life is assayed at 80 degrees C. Preferably, the sample is heated for 1-10 minutes at 80°C or higher. The half life value is then calculated by measuring the residual amylase activity, by any of the methods described here. Preferably, a half life assay is conducted as described in more detail in the Examples.

Preferably, the PS4 variants described here are active during baking and hydrolyse starch during and after the gelatinization of the starch granules which starts at temperatures of about 55°C. The more thermostable the non-maltogenic exoamylase is the longer time it can be active and thus the more antistaling effect it will provide. However, during baking above temperatures of about 85°C, enzyme inactivation can take place. If this happens, the non-maltogenic exoamylase may be gradually inactivated so that there is substantially no activity after the baking process in the final bread. Therefore preferentially the non-maltogenic exoamylases suitable for use as described have an optimum temperature above 50°C and below 98°C.

The thermostability of the PS4 variants described here can be improved by using protein engineering to become more thermostable and thus better suited for the uses described here; we therefore encompass the use of PS4 variants modified to become more thermostable by protein engineering.

Preferably, the PS4 variant polypeptide is pH stable; more preferably, it has a higher pH stability than its cognate parent polypeptide. As used herein the term 'pH stable' relates to the ability of the enzyme to retain activity over a wide range of pHs. Preferably, the PS4 variant polypeptide is capable of degrading starch at a pH of from about 5 to about 10.5. In one embodiment, the degree of pH stability may be assayed by measuring the half life of the enzyme in specific pH conditions. In another embodiment, the degree of pH stability may be assayed by measuring the activity or specific activity of the enzyme in specific pH conditions. The specific pH conditions may be any pH from pH5 to pH10.5.

Thus, the PS4 variant polypeptide may have a longer half life, or a higher activity (depending on the assay) when compared to the parent polypeptide under identical conditions. The PS4 variant polypeptides may have 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or longer half life when compared to their parent
795 polypeptides under identical pH conditions. Alternatively, or in addition, they may have such higher activity when compared to the parent polypeptide under identical pH conditions.

EXO-SPECIFICITY

It is known that some non-maltogenic exoamylases can have some degree of
800 endoamylase activity. In some cases, this type of activity may need to be reduced or eliminated since endoamylase activity can possibly negatively effect the quality of the final bread product by producing a sticky or gummy crumb due to the accumulation of branched dextrans.

Exo-specificity can usefully be measured by determining the ratio of total amylase
805 activity to the total endoamylase activity. This ratio is referred to in this document as a "Exo-specificity index". In preferred embodiments, an enzyme is considered an exoamylase if it has a exo-specificity index of 20 or more, i.e., its total amylase activity (including exo-amylase activity) is 20 times or more greater than its endoamylase activity. In highly preferred embodiments, the exo-specificity index of exoamylases is 30 or more,
810 40 or more, 50 or more, 60 or more, 70 or more, 80 or more, 90 or more, or 100 or more. In highly preferred embodiments, the exo-specificity index is 150 or more, 200 or more, 300 or more, or 400 or more.

The total amylase activity and the endoamylase activity may be measured by any means known in the art. For example, the total amylase activity may be measured by
815 assaying the total number of reducing ends released from a starch substrate. Alternatively, the use of a Betamyl assay is described in further detail in the Examples, and for convenience, amylase activity as assayed in the Examples is described in terms of "Betamyl Units" in the Tables and Figures.

Endoamylase activity may be assayed by use of a Phadebas Kit (Pharmacia and
820 Upjohn). This makes use of a blue labelled crosslinked starch (labelled with an azo dye); only internal cuts in the starch molecule release label, while external cuts do not do so.

Release of dye may be measured by spectrophotometry. Accordingly, the Phadebas Kit measures endoamylase activity, and for convenience, the results of such an assay (described in the Examples) are referred to in this document as "Phadebas units".

825 In a highly preferred embodiment, therefore, the exo-specificity index is expressed in terms of Betamyl Units / Phadebas Units.

Exo-specificity may also be assayed according to the methods described in the prior art, for example, in our International Patent Publication Number WO99/50399. This measures exo-specificity by way of a ratio between the endoamylase activity to the
830 exoamylase activity. Thus, in a preferred aspect, the PS4 variants described here will have less than 0.5 endoamylase units (EAU) per unit of exoamylase activity. Preferably the non-maltogenic exoamylases which are suitable for use according to the present invention have less than 0.05 EAU per unit of exoamylase activity and more preferably less than 0.01 EAU per unit of exoamylase activity.

835 The PS4 variants described here will preferably have exospecificity, for example measured by exo-specificity indices, as described above, consistent with their being exoamylases. Moreover, they preferably have higher or increased exospecificity when compared to the parent enzymes or polypeptides from which they are derived. Thus, for example, the PS4 variant polypeptides may have 10%, 20%, 30%, 40%, 50%, 60%, 70%,
840 80%, 90%, 100%, 200% or higher exo-specificity index when compared to their parent polypeptides, preferably under identical conditions. They may have 1.5x or higher, 2x or higher, 5 x or higher, 10 x or higher, 50 x or higher, 100 x or higher, when compared to their parent polypeptides, preferably under identical conditions.

USES OF PS4 VARIANT POLYPEPTIDES AND NUCLEIC ACIDS

845 The PS4 variant polypeptides, nucleic acids, host cells, expression vectors, etc, may be used in any application for which an amylase may be used. In particular, they may be used to substitute for any non-maltogenic exoamylase. They may be used to supplement amylase or non-maltogenic exoamylase activity, whether alone or in combination with other known amylases or non-maltogenic exoamylases.

850 The PS4 variant sequences described here may be used in various applications in the food industry – such as in bakery and drink products, they may also be used in other

applications such as a pharmaceutical composition, or even in the chemical industry. In particular, the PS4 variant polypeptides and nucleic acids are useful for various industrial applications including baking (as disclosed in WO 99/50399) and flour standardisation
855 (volume enhancement or improvement). They may be used to produce maltotetraose from starch and other substrates.

The PS4 variant polypeptides may be used to enhance the volume of bakery products such as bread. While not wishing to be bound by any particular theory, we believe that this results from the reduction in viscosity of the dough during heating (such
860 as baking) as a result of the exoamylase shortening amylose molecules. This enables the carbon dioxide generated by fermentation to increase the size of the bread with less hindrance.

Thus, food products comprising or treated with PS4 variant polypeptides are expanded in volume when compared to products which have not been so treated, or treated
865 with parent polypeptides. In other words, the food products have a larger volume of air per volume of food product. Alternatively, or in addition, the food products treated with PS4 variant polypeptides have a lower density, or weight (or mass) per volume ratio. In particularly preferred embodiments, the PS4 variant polypeptides are used to enhance the volume of bread. Volume enhancement or expansion is beneficial because it reduces the
870 gumminess or starchiness of foods. Light foods are preferred by consumers, and the customer experience is enhanced. In preferred embodiments, the use of PS4 variant polypeptides enhances the volume by 10%, 20%, 30% 40%, 50% or more.

The use of PS4 variant polypeptides to increase the volume of foods is described in detail in the Examples.

875 **FOOD USES**

The PS4 variant polypeptides and nucleic acids described here may be used as – or in the preparation of – a food. In particular, they may be added to a food, i.e., as a food additive. The term “food” is intended to include both prepared food, as well as an ingredient for a food, such as a flour. In a preferred aspect, the food is for human
880 consumption. The food may be in the form of a solution or as a solid – depending on the use and/or the mode of application and/or the mode of administration.

The PS4 variant polypeptides and nucleic acids may be used as a food ingredient. As used herein the term "food ingredient" includes a formulation, which is or can be added to functional foods or foodstuffs and includes formulations which can be used at low levels in a wide variety of products that require, for example, acidifying or emulsifying. The food ingredient may be in the form of a solution or as a solid – depending on the use and/or the mode of application and/or the mode of administration.

The PS4 variant polypeptides and nucleic acids disclosed here may be – or may be added to – food supplements. The PS4 variant polypeptides and nucleic acids disclosed here may be – or may be added to – functional foods. As used herein, the term "functional food" means food which is capable of providing not only a nutritional effect and/or a taste satisfaction, but is also capable of delivering a further beneficial effect to consumer. Although there is no legal definition of a functional food, most of the parties with an interest in this area agree that they are foods marketed as having specific health effects.

The PS4 variant polypeptides may also be used in the manufacture of a food product or a foodstuff. Typical foodstuffs include dairy products, meat products, poultry products, fish products and dough products. The dough product may be any processed dough product, including fried, deep fried, roasted, baked, steamed and boiled doughs, such as steamed bread and rice cakes. In highly preferred embodiments, the food product is a bakery product.

Preferably, the foodstuff is a bakery product. Typical bakery (baked) products include bread – such as loaves, rolls, buns, pizza bases etc. pastry, pretzels, tortillas, cakes, cookies, biscuits, crackers etc.

RETROGRADATION / STALING

We describe the use of PS4 variant proteins that are capable of retarding the staling of starch media, such as starch gels. The PS4 variant polypeptides are especially capable of retarding the detrimental retrogradation of starch.

Most starch granules are composed of a mixture of two polymers: an essentially linear amylose and a highly branched amylopectin. Amylopectin is a very large, branched molecule consisting of chains of α -D-glucopyranosyl units joined by (1-4) linkages, wherein said chains are attached by α -D-(1-6) linkages to form branches. Amylopectin is

present in all natural starches, constituting about 75% of most common starches. Amylose is essentially a linear chain of (1-4) linked α -D-glucopyranosyl units having few α -D-(1-6) branches. Most starches contain about 25% amylose.

915 Starch granules heated in the presence of water undergo an order-disorder phase transition called gelatinization, where liquid is taken up by the swelling granules. Gelatinization temperatures vary for different starches. Upon cooling of freshly baked bread the amylose fraction, within hours, retrogrades to develop a network. This process is beneficial in that it creates a desirable crumb structure with a low degree of firmness and
920 improved slicing properties. More gradually crystallisation of amylopectin takes place within the gelatinised starch granules during the days after baking. In this process amylopectin is believed to reinforce the amylose network in which the starch granules are embedded. This reinforcement leads to increased firmness of the bread crumb. This reinforcement is one of the main causes of bread staling.

925 It is known that the quality of baked products gradually deteriorates during storage. As a consequence of starch recrystallisation (also called retrogradation), the water-holding capacity of the crumb is changed with important implications on the organoleptic and dietary properties. The crumb loses softness and elasticity and becomes firm and crumbly. The increase in crumb firmness is often used as a measure of the staling process of bread.

930 The rate of detrimental retrogradation of amylopectin depends on the length of the side chains of amylopectin. Thus, enzymatic hydrolysis of the amylopectin side chains, for example, by PS4 variant polypeptides having non-maltogenic exoamylase activity, can markedly reduce their crystallisation tendencies.

 Accordingly, the use of PS4 variant polypeptides as described here when added to
935 the starch at any stage of its processing into a food product, e.g., before during or after baking into bread can retard or impede or slow down the retrogradation. Such use is described in further detail below.

ASSAYS FOR MEASUREMENT OF RETROGRADATION (INC. STALING)

 For evaluation of the antistaling effect of the PS4 variant polypeptides having non-
940 maltogenic exoamylase activity described here, the crumb firmness can be measured 1, 3

and 7 days after baking by means of an Instron 4301 Universal Food Texture Analyzer or similar equipment known in the art.

Another method used traditionally in the art and which is used to evaluate the effect on starch retrogradation of a PS4 variant polypeptide having non-maltogenic
945 exoamylase activity is based on DSC (differential scanning calorimetry). Here, the melting enthalpy of retrograded amylopectin in bread crumb or crumb from a model system dough baked with or without enzymes (control) is measured. The DSC equipment applied in the described examples is a Mettler-Toledo DSC 820 run with a temperature gradient of 10°C per min. from 20 to 95°C. For preparation of the samples 10-20 mg of crumb are weighed
950 and transferred into Mettler-Toledo aluminium pans which then are hermetically sealed.

The model system doughs used in the described examples contain standard wheat flour and optimal amounts of water or buffer with or without the non-maltogenic PS4 variant exoamylase. They are mixed in a 10 or 50 g Brabender Farinograph for 6 or 7 min., respectively. Samples of the doughs are placed in glass test tubes (15*0.8 cm) with a
955 lid. These test tubes are subjected to a baking process in a water bath starting with 30 min. incubation at 33°C followed by heating from 33 to 95°C with a gradient of 1.1°C per min. and finally a 5 min. incubation at 95°C. Subsequently, the tubes are stored in a thermostat at 20°C prior to DSC analysis.

In preferred embodiments, the PS4 variants described here have a reduced melting
960 enthalpy, compared to the control. In highly preferred embodiments, the PS4 variants have a 10% or more reduced melting enthalpy. Preferably, they have a 20% or more, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more reduced melting enthalpy when compared to the control.

| | DSC (J/g) |
|---------|-----------|
| Control | 2,29 |
| 0,5 D34 | 1,91 |
| 1 D34 | 1,54 |
| 2 D34 | 1,14 |

965 Table 2

The above Table 2 shows DSC values of model dough systems prepared with different doses of PSac-D34 after 7 days of storage. 0.5, 1 and 2 parts per million (or microgram per gram) of flour are tested.

PREPARATION OF STARCH PRODUCTS

970 We provide the use of PS4 variant polypeptides in the preparation of food products, in particular, starch products. The method comprises forming the starch product by adding a non-maltogenic exoamylase enzyme such as a PS4 variant polypeptide, to a starch medium. If the starch medium is a dough, then the dough is prepared by mixing together flour, water, the non-maltogenic exoamylase which is a PS4 variant polypeptide
975 and optionally other possible ingredients and additives.

The term "starch" should be taken to mean starch *per se* or a component thereof, especially amylopectin. The term "starch medium" means any suitable medium comprising starch. The term "starch product" means any product that contains or is based on or is derived from starch. Preferably, the starch product contains or is based on or is
980 derived from starch obtained from wheat flour. The term "flour" as used herein is a synonym for the finely-ground meal of wheat or other grain. Preferably, however, the term means flour obtained from wheat *per se* and not from another grain. Thus, and unless otherwise expressed, references to "wheat flour" as used herein preferably mean references to wheat flour *per se* as well as to wheat flour when present in a medium, such
985 as a dough.

A preferred flour is wheat flour or rye flour or mixtures of wheat and rye flour. However, dough comprising flour derived from other types of cereals such as for example from rice, maize, barley, and durra are also contemplated. Preferably, the starch product is a bakery product. More preferably, the starch product is a bread product. Even more
990 preferably, the starch product is a baked farinaceous bread product. The term "baked farinaceous bread product" refers to any baked product based on a dough obtainable by mixing flour, water, and a leavening agent under dough forming conditions. Further components can of course be added to the dough mixture.

Thus, if the starch product is a baked farinaceous bread product, then the process
995 comprises mixing - in any suitable order - flour, water, and a leavening agent under dough forming conditions and further adding a PS4 variant polypeptide, optionally in the form of a premix. The leavening agent may be a chemical leavening agent such as sodium bicarbonate or any strain of *Saccharomyces cerevisiae* (Baker's Yeast).

The PS4 variant non-maltogenic exoamylase can be added together with any dough ingredient including the water or dough ingredient mixture or with any additive or additive mixture. The dough can be prepared by any conventional dough preparation method common in the baking industry or in any other industry making flour dough based products.

Baking of farinaceous bread products such as for example white bread, bread made from bolted rye flour and wheat flour, rolls and the like is typically accomplished by baking the bread dough at oven temperatures in the range of from 180 to 250°C for about 15 to 60 minutes. During the baking process a steep temperature gradient (200 → 120°C) is prevailing in the outer dough layers where the characteristic crust of the baked product is developed. However, owing to heat consumption due to steam generation, the temperature in the crumb is only close to 100°C at the end of the baking process.

We therefore describe a process for making a bread product comprising: (a) providing a starch medium; (b) adding to the starch medium a PS4 variant polypeptide as described in this document; and (c) applying heat to the starch medium during or after step (b) to produce a bread product. We also describe a process for making a bread product comprising adding to a starch medium a PS4 variant polypeptide as described.

The non-maltogenic exoamylase PS4 variant polypeptide can be added as a liquid preparation or as a dry pulverulent composition either comprising the enzyme as the sole active component or in admixture with one or more additional dough ingredient or dough additive.

1020 IMPROVING COMPOSITION

We describe improver compositions, which include bread improving compositions and dough improving compositions. These comprise a PS4 variant polypeptide, optionally together with a further ingredient, or a further enzyme, or both.

We also provide for the use of such a bread and dough improving compositions in baking. In a further aspect, we provide a baked product or dough obtained from the bread improving composition or dough improving composition. In another aspect, we describe a baked product or dough obtained from the use of a bread improving composition or a dough improving composition.

DOUGH PREPARATION

1030 A dough may be prepared by admixing flour, water, a dough improving composition comprising PS4 variant polypeptide (as described above) and optionally other ingredients and additives.

1035 The dough improving composition can be added together with any dough ingredient including the flour, water or optional other ingredients or additives. The dough improving composition can be added before the flour or water or optional other ingredients and additives. The dough improving composition can be added after the flour or water, or optional other ingredients and additives. The dough can be prepared by any conventional dough preparation method common in the baking industry or in any other industry making flour dough based products.

1040 The dough improving composition can be added as a liquid preparation or in the form of a dry powder composition either comprising the composition as the sole active component or in admixture with one or more other dough ingredients or additive.

1045 The amount of the PS4 variant polypeptide non-maltogenic exoamylase that is added is normally in an amount which results in the presence in the finished dough of 50 to 100,000 units per kg of flour, preferably 100 to 50,000 units per kg of flour. Preferably, the amount is in the range of 200 to 20,000 units per kg of flour.

1050 In the present context, 1 unit of the non-maltogenic exoamylase is defined as the amount of enzyme which releases hydrolysis products equivalent to 1 μ mol of reducing sugar per min. when incubated at 50 degrees C in a test tube with 4 ml of 10 mg/ml waxy maize starch in 50 mM MES, 2 mM calcium chloride, pH 6.0 as described hereinafter.

 The dough as described here generally comprises wheat meal or wheat flour and/or other types of meal, flour or starch such as corn flour, corn starch, maize flour, rice flour, rye meal, rye flour, oat flour, oat meal, soy flour, sorghum meal, sorghum flour, potato meal, potato flour or potato starch. The dough may be fresh, frozen, or part-baked.

1055 The dough may be a leavened dough or a dough to be subjected to leavening. The dough may be leavened in various ways, such as by adding chemical leavening agents, e.g., sodium bicarbonate or by adding a leaven (fermenting dough), but it is preferred to

leaven the dough by adding a suitable yeast culture, such as a culture of *Saccharomyces cerevisiae* (baker's yeast), e.g. a commercially available strain of *S. cerevisiae*.

1060 The dough may comprise fat such as granulated fat or shortening. The dough may further comprise a further emulsifier such as mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides, polyoxyethylene stearates, or lysolecithin.

1065 We also describe a pre-mix comprising flour together with the combination as described herein. The pre-mix may contain other dough-improving and/or bread-improving additives, e.g. any of the additives, including enzymes, mentioned herein.

FURTHER DOUGH ADDITIVES OR INGREDIENTS

1070 In order to improve further the properties of the baked product and impart distinctive qualities to the baked product further dough ingredients and/or dough additives may be incorporated into the dough. Typically, such further added components may include dough ingredients such as salt, grains, fats and oils, sugar or sweetener, dietary fibres, protein sources such as milk powder, gluten soy or eggs and dough additives such as emulsifiers, other enzymes, hydrocolloids, flavouring agents, oxidising agents, minerals and vitamins

1075 The emulsifiers are useful as dough strengtheners and crumb softeners. As dough strengtheners, the emulsifiers can provide tolerance with regard to resting time and tolerance to shock during the proofing. Furthermore, dough strengtheners will improve the tolerance of a given dough to variations in the fermentation time. Most dough strengtheners also improve on the oven spring which means the increase in volume from
1080 the proofed to the baked goods. Lastly, dough strengtheners will emulsify any fats present in the recipe mixture.

1085 Suitable emulsifiers include lecithin, polyoxyethylene stearat, mono- and diglycerides of edible fatty acids, acetic acid esters of mono- and diglycerides of edible fatty acids, lactic acid esters of mono- and diglycerides of edible fatty acids, citric acid esters of mono- and diglycerides of edible fatty acids, diacetyl tartaric acid esters of mono- and diglycerides of edible fatty acids, sucrose esters of edible fatty acids, sodium stearyl-2-lactylate, and calcium stearyl-2-lactylate.

The further dough additive or ingredient can be added together with any dough ingredient including the flour, water or optional other ingredients or additives, or the
1090 dough improving composition. The further dough additive or ingredient can be added before the flour, water, optional other ingredients and additives or the dough improving composition. The further dough additive or ingredient can be added after the flour, water, optional other ingredients and additives or the dough improving composition.

The further dough additive or ingredient may conveniently be a liquid preparation.
1095 However, the further dough additive or ingredient may be conveniently in the form of a dry composition.

Preferably the further dough additive or ingredient is at least 1% the weight of the flour component of dough. More preferably, the further dough additive or ingredient is at least 2%, preferably at least 3%, preferably at least 4%, preferably at least 5%, preferably
1100 at least 6%. If the additive is a fat, then typically the fat may be present in an amount of from 1 to 5%, typically 1 to 3%, more typically about 2%.

FURTHER ENZYME

In addition to the PS4 variant polypeptides, one or more further enzymes may be used, for example added to the food, dough preparation, foodstuff or starch composition.

1105 Further enzymes that may be added to the dough include oxidoreductases, hydrolases, such as lipases and esterases as well as glycosidases like α -amylase, pullulanase, and xylanase. Oxidoreductases, such as for example glucose oxidase and hexose oxidase, can be used for dough strengthening and control of volume of the baked products and xylanases and other hemicellulases may be added to improve dough handling
1110 properties, crumb softness and bread volume. Lipases are useful as dough strengtheners and crumb softeners and α -amylases and other amylolytic enzymes may be incorporated into the dough to control bread volume and further reduce crumb firmness.

Further enzymes that may be used may be selected from the group consisting of a cellulase, a hemicellulase, a starch degrading enzyme, a protease, a lipoxygenase.

1115 Examples of useful oxidoreductases include oxidises such as maltose oxidising enzyme, a glucose oxidase (EC 1.1.3.4), carbohydrate oxidase, glycerol oxidase, pyranose oxidase, galactose oxidase (EC 1.1.3.10) and hexose oxidase (EC 1.1.3.5).

Among starch degrading enzymes, amylases are particularly useful as dough improving additives. α -amylase breaks down starch into dextrins which are further
1120 broken down by β -amylase to maltose. Other useful starch degrading enzymes which may be added to a dough composition include glucoamylases and pullulanases.

Preferably, the further enzyme is at least a xylanase and/or at least an amylase. The term "xylanase" as used herein refers to xylanases (EC 3.2.1.32) which hydrolyse xylosidic linkages.

1125 The term "amylase" as used herein refers to amylases such as α -amylases (EC 3.2.1.1), β -amylases (EC 3.2.1.2) and γ -amylases (EC 3.2.1.3).

The further enzyme can be added together with any dough ingredient including the flour, water or optional other ingredients or additives, or the dough improving composition. The further enzyme can be added before the flour, water, and optionally
1130 other ingredients and additives or the dough improving composition. The further enzyme can be added after the flour, water, and optionally other ingredients and additives or the dough improving composition. The further enzyme may conveniently be a liquid preparation. However, the composition may be conveniently in the form of a dry composition.

1135 Some enzymes of the dough improving composition are capable of interacting with each other under the dough conditions to an extent where the effect on improvement of the rheological and/or machineability properties of a flour dough and/or the quality of the product made from dough by the enzymes is not only additive, but the effect is synergistic.

In relation to improvement of the product made from dough (finished product), it
1140 may be found that the combination results in a substantial synergistic effect in respect to crumb structure. Also, with respect to the specific volume of baked product a synergistic effect may be found.

The further enzyme may be a lipase (EC 3.1.1) capable of hydrolysing carboxylic ester bonds to release carboxylate. Examples of lipases include but are not limited to
1145 triacylglycerol lipase (EC 3.1.1.3), galactolipase (EC 3.1.1.26), phospholipase A1 (EC 3.1.1.32, phospholipase A2 (EC 3.1.1.4) and lipoprotein lipase A2 (EC 3.1.1.34).

AMYLASE COMBINATIONS

We disclose in particular combinations of PS4 variant polypeptides with amylases, in particular, maltogenic amylases. Maltogenic alpha-amylase (glucan 1,4-a-
1150 maltohydrolase, E.C. 3.2.1.133) is able to hydrolyze amylose and amylopectin to maltose in the alpha-configuration.

A maltogenic alpha-amylase from *Bacillus* (EP 120 693) is commercially available under the trade name Novamyl (Novo Nordisk A/S, Denmark) and is widely used in the baking industry as an anti-staling agent due to its ability to reduce retrogradation of starch.
1155 Novamyl is described in detail in International Patent Publication WO 91/04669. The maltogenic alpha-amylase Novamyl shares several characteristics with cyclodextrin glucanotransferases (CGTases), including sequence homology (Henrissat B, Bairoch A; *Biochem. J.*, 316, 695-696 (1996)) and formation of transglycosylation products (Christophersen, C., et al., 1997, *Starch*, vol. 50, No. 1, 39-45).

1160 In highly preferred embodiments, we disclose combinations comprising PS4 variant polypeptides together with Novamyl or any of its variants. Such combinations are useful for food production such as baking. The Novamyl may in particular comprise Novamyl 1500 MG.

Other documents describing Novamyl and its uses include Christophersen, C.,
1165 Pedersen, S., and Christensen, T., (1993) Method for production of maltose and a limit dextrin, the limit dextrin, and use of the limit dextrin. Denmark, and WO 95/10627. It is further described in U.S. Pat. No. 4,598,048 and U.S. Pat. No. 4,604,355. Each of these documents is hereby incorporated by reference, and any of the Novamyl polypeptides described therein may be used in combinations with any of the PS4 variant polypeptides
1170 described here.

Variants, homologues, and mutants of Novamyl may be used for the combinations, provided they retain alpha amylase activity. For example, any of the Novamyl variants disclosed in US Patent Number 6,162,628, the entire disclosure of which is hereby incorporated by reference, may be used in combination with the PS4 variant polypeptides
1175 described here. In particular, any of the polypeptides described in that document, specifically variants of SEQ ID NO:1 of US 6,162,628 at any one or more positions corresponding to Q13, I16, D17, N26, N28, P29, A30, S32, Y33, G34, L35, K40, M45,

P73, V74, D76 N77, D79, N86, R95, N99, I100, H103, Q119, N120, N131, S141, T142, A148, N152, A163, H169, N171, G172, I174, N176, N187, F188, A192, Q201, N203, 1180 H220, N234, G236, Q247, K249, D261, N266, L268, R272, N275, N276, V279, N280, V281, D285, N287, F297, Q299, N305, K316, N320, L321, N327, A341, N342, A348, Q365, N371, N375, M378, G397, A381, F389, N401, A403, K425, N436, S442, N454, N468, N474, S479, A483, A486, V487, S493, T494, S495, A496, S497, A498, Q500, N507, I510, N513, K520, Q526, A555, A564, S573, N575, Q581, S583, F586, K589, 1185 N595, G618, N621, Q624, A629, F636, K645, N664 and/or T681 may be used.

AMINO ACID SEQUENCES

The invention makes use of a PS4 variant nucleic acid, and the amino acid sequences of such PS4 variant nucleic acids are encompassed by the methods and compositions described here.

1190 As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide". In some instances, the term "amino acid sequence" is synonymous with the term "enzyme".

The amino acid sequence may be prepared/isolated from a suitable source, or it 1195 may be made synthetically or it may be prepared by use of recombinant DNA techniques.

The PS4 variant enzyme described here may be used in conjunction with other enzymes. Thus we further disclose a combination of enzymes wherein the combination comprises a PS4 variant polypeptide enzyme described here and another enzyme, which itself may be another PS4 variant polypeptide enzyme.

1200 PS4 VARIANT NUCLEOTIDE SEQUENCE

As noted above, we disclose nucleotide sequences encoding the PS4 variant enzymes having the specific properties described.

The term "nucleotide sequence" or "nucleic acid sequence" as used herein refers to an oligonucleotide sequence or polynucleotide sequence, and variant, homologues, 1205 fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-stranded or single-stranded whether representing the sense or anti-sense strand.

The term "nucleotide sequence" as used in this document includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA
1210 sequence coding for a PS4 variant polypeptide.

Typically, the PS4 variant nucleotide sequence is prepared using recombinant DNA techniques (i.e. recombinant DNA). However, in an alternative embodiment, the nucleotide sequence could be synthesised, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al.*, (1980) *Nuc Acids Res Symp Ser* 215-23
1215 and Horn T *et al.*, (1980) *Nuc Acids Res Symp Ser* 225-232).

PREPARATION OF NUCLEIC ACID SEQUENCES

A nucleotide sequence encoding either an enzyme which has the specific properties as defined herein (e.g., a PS4 variant polypeptide) or an enzyme which is suitable for modification, such as a parent enzyme, may be identified and/or isolated and/or purified
1220 from any cell or organism producing said enzyme. Various methods are well known within the art for the identification and/or isolation and/or purification of nucleotide sequences. By way of example, PCR amplification techniques to prepare more of a sequence may be used once a suitable sequence has been identified and/or isolated and/or purified.

By way of further example, a genomic DNA and/or cDNA library may be constructed using chromosomal DNA or messenger RNA from the organism producing the enzyme. If the amino acid sequence of the enzyme or a part of the amino acid sequence of the enzyme is known, labelled oligonucleotide probes may be synthesised and used to identify enzyme-encoding clones from the genomic library prepared from the
1230 organism. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known enzyme gene could be used to identify enzyme-encoding clones. In the latter case, hybridisation and washing conditions of lower stringency are used.

Alternatively, enzyme-encoding clones could be identified by inserting fragments
1235 of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar plates containing a substrate for enzyme (i.e. maltose), thereby allowing clones expressing the enzyme to be identified.

1240 In a yet further alternative, the nucleotide sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by Beucage S.L. *et al.*, (1981) *Tetrahedron Letters* 22, p 1859-1869, or the method described by Matthes *et al.*, (1984) *EMBO J.* 3, p 801-805. In the phosphoroamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA synthesiser, purified, annealed, ligated and cloned in appropriate vectors.

1245 The nucleotide sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin, or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate) in accordance with standard techniques. Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain
1250 reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in Saiki R K *et al.*, (*Science* (1988) 239, pp 487-491).

VARIANTS/HOMOLOGUES/DERIVATIVES

We further describe the use of variants, homologues and derivatives of any amino acid sequence of an enzyme or of any nucleotide sequence encoding such an enzyme, such
1255 as a PS4 variant polypeptide or a PS4 variant nucleic acid. Unless the context dictates otherwise, the term "PS4 variant nucleic acid" should be taken to include each of the nucleic acid entities described below, and the term "PS4 variant polypeptide" should likewise be taken to include each of the polypeptide or amino acid entities described below.

Here, the term "homologue" means an entity having a certain homology with the
1260 subject amino acid sequences and the subject nucleotide sequences. Here, the term "homology" can be equated with "identity".

In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 80, 85 or 90% identical, preferably at least 95, 96, 97, 98 or 99% identical to the subject sequence.

1265 Preferably, therefore, the PS4 variant polypeptides for use in the methods and compositions described here comprise polypeptides having at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least

96%, at least 97%, at least 98%, or at least 99%, homology or sequence identity with the sequences set out in the Sequence Listings.

1270 Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of this document it is preferred to express homology in terms of sequence identity.

1275 In the present context, an homologous sequence is taken to include a nucleotide sequence which may be at least 75, 80, 85 or 90% identical, preferably at least 95, 96, 97, 98 or 99% identical to a nucleotide sequence encoding a PS4 variant polypeptide enzyme (such as a PS4 variant nucleic acid). Typically, the homologues will comprise the same sequences that code for the active sites etc as the subject sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar
1280 chemical properties/functions), in the context of this document it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

1285 % homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

1290 Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce
1295 optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux *et al* 1984 Nuc. Acids Research 12 p387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *Short Protocols in Molecular Biology*, 4th Ed – Chapter 18), FASTA (Altschul *et al.*, 1990 *J. Mol. Biol.* 403-410) and the GENWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999, *Short Protocols in Molecular Biology*, pages 7-58 to 7-60).

However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see *FEMS Microbiol Lett* 1999 174(2): 247-50; *FEMS Microbiol Lett* 1999 177(1): 187-8 and tatiana@ncbi.nlm.nih.gov).

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for

1330 further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASISTM (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *Gene* 73(1), 237-244).

1335 Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance.

1340 Deliberate amino acid substitutions may be made on the basis of similarity in amino acid properties (such as polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues) and it is therefore useful to group amino acids together in functional groups. Amino acids can be grouped together based on the properties of their side chain alone. However it is more useful to include mutation data as well. The sets of

1345 amino acids thus derived are likely to be conserved for structural reasons. These sets can be described in the form of a Venn diagram (Livingstone C.D. and Barton G.J. (1993) "Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation" *Comput. Appl Biosci.* 9: 745-756)(Taylor W.R. (1986) "The classification of amino acid conservation" *J.Theor.Biol.* 119; 205-218). Conservative substitutions may be

1350 made, for example according to the table below which describes a generally accepted Venn diagram grouping of amino acids.

| Set | | Sub-set | |
|-------------|-------------------------|--------------------|-----------|
| Hydrophobic | F W Y H K M I L V A G C | Aromatic | F W Y H |
| | | Aliphatic | I L V |
| Polar | W Y H K R E D C S T N Q | Charged | H K R E D |
| | | Positively charged | H K R |
| | | Negatively charged | E D |
| Small | V C A G S P T N D | Tiny | A G S |

We further disclose sequences comprising homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β -alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α -carbon substituent group is on the residue's nitrogen atom rather than the α -carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ *et al.*, *PNAS* (1992) 89(20), 9367-9371 and Horwell DC, *Trends Biotechnol.* (1995) 13(4), 132-134.

The nucleotide sequences described here, and suitable for use in the methods and compositions described here (such as PS4 variant nucleic acids) may include within them

synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of this document, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of nucleotide sequences.

We further describe the use of nucleotide sequences that are complementary to the sequences presented herein, or any derivative, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify similar coding sequences in other organisms etc.

Polynucleotides which are not 100% homologous to the PS4 variant sequences may be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other homologues may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other species, and probing such libraries with probes comprising all or part of any one of the sequences in the attached sequence listings under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences described here.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences. This may be useful where for example silent codon sequence
1405 changes are required to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

The polynucleotides (nucleotide sequences) such as the PS4 variant nucleic acids
1410 described in this document may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also
1415 encompassed by the term polynucleotides.

Polynucleotides such as DNA polynucleotides and probes may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques. In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide
1420 at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA
1425 can be cloned into a suitable cloning vector. Preferably, the variant sequences etc. are at least as biologically active as the sequences presented herein.

As used herein "biologically active" refers to a sequence having a similar structural function (but not necessarily to the same degree), and/or similar regulatory function (but not necessarily to the same degree), and/or similar biochemical function (but not
1430 necessarily to the same degree) of the naturally occurring sequence.

HYBRIDISATION

We further describe sequences that are complementary to the nucleic acid sequences of PS4 variants or sequences that are capable of hybridising either to the PS4 variant sequences or to sequences that are complementary thereto.

1435 The term “hybridisation” as used herein shall include “the process by which a strand of nucleic acid joins with a complementary strand through base pairing” as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies. Therefore, we disclose the use of nucleotide sequences that are capable of hybridising to the sequences that are complementary to the sequences presented herein, or
1440 any derivative, fragment or derivative thereof.

The term “variant” also encompasses sequences that are complementary to sequences that are capable of hybridising to the nucleotide sequences presented herein.

Preferably, the term “variant” encompasses sequences that are complementary to sequences that are capable of hybridising under stringent conditions (e.g. 50°C and
1445 0.2xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na₃citrate pH 7.0}) to the nucleotide sequences presented herein. More preferably, the term “variant” encompasses sequences that are complementary to sequences that are capable of hybridising under high stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na₃citrate pH 7.0}) to the nucleotide sequences presented herein.

1450 We further disclose nucleotide sequences that can hybridise to the nucleotide sequences of PS4 variants (including complementary sequences of those presented herein), as well as nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences of PS4 variants (including complementary sequences of those presented herein). We further describe polynucleotide sequences that are capable of
1455 hybridising to the nucleotide sequences presented herein under conditions of intermediate to maximal stringency.

In a preferred aspect, we disclose nucleotide sequences that can hybridise to the nucleotide sequence of a PS4 variant nucleic acid, or the complement thereof, under stringent conditions (e.g. 50°C and 0.2xSSC). More preferably, the nucleotide sequences

1460 can hybridise to the nucleotide sequence of a PS4 variant, or the complement thereof,
under high stringent conditions (e.g. 65°C and 0.1xSSC).

SITE-DIRECTED MUTAGENESIS

Once an enzyme-encoding nucleotide sequence has been isolated, or a putative
enzyme-encoding nucleotide sequence has been identified, it may be desirable to mutate
1465 the sequence in order to prepare an enzyme. Accordingly, a PS4 variant sequence may be
prepared from a parent sequence. Mutations may be introduced using synthetic
oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the
desired mutation sites.

A suitable method is disclosed in Morinaga *et al.*, (*Biotechnology* (1984) 2, p646-
1470 649). Another method of introducing mutations into enzyme-encoding nucleotide
sequences is described in Nelson and Long (*Analytical Biochemistry* (1989), 180, p 147-
151). A further method is described in Sarkar and Sommer (*Biotechniques* (1990), 8,
p404-407 – “The megaprimer method of site directed mutagenesis”).

In one aspect the sequence for use in the methods and compositions described here
1475 is a recombinant sequence – i.e. a sequence that has been prepared using recombinant
DNA techniques. These recombinant DNA techniques are within the capabilities of a
person of ordinary skill in the art. Such techniques are explained in the literature, for
example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A
Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press.

1480 In one aspect the sequence for use in the methods and compositions described here
is a synthetic sequence – i.e. a sequence that has been prepared by *in vitro* chemical or
enzymatic synthesis. It includes, but is not limited to, sequences made with optimal codon
usage for host organisms - such as the methylotrophic yeasts *Pichia* and *Hansenula*.

The nucleotide sequence for use in the methods and compositions described here
1485 may be incorporated into a recombinant replicable vector. The vector may be used to
replicate and express the nucleotide sequence, in enzyme form, in and/or from a
compatible host cell. Expression may be controlled using control sequences eg. regulatory
sequences. The enzyme produced by a host recombinant cell by expression of the
nucleotide sequence may be secreted or may be contained intracellularly depending on the

1490 sequence and/or the vector used. The coding sequences may be designed with signal sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

EXPRESSION OF PS4 NUCLEIC ACIDS AND POLYPEPTIDES

The PS4 polynucleotides and nucleic acids may include DNA and RNA of both
1495 synthetic and natural origin which DNA or RNA may contain modified or unmodified deoxy- or dideoxy- nucleotides or ribonucleotides or analogs thereof. The PS4 nucleic acid may exist as single- or double-stranded DNA or RNA, an RNA/DNA heteroduplex or an RNA/DNA copolymer, wherein the term "copolymer" refers to a single nucleic acid strand that comprises both ribonucleotides and deoxyribonucleotides. The PS4 nucleic acid may
1500 even be codon optimised to further increase expression.

The term "synthetic", as used herein, is defined as that which is produced by *in vitro* chemical or enzymatic synthesis. It includes but is not limited to PS4 nucleic acids made with optimal codon usage for host organisms such as the the methylotrophic yeasts *Pichia* and *Hansenula*.

1505 Polynucleotides, for example variant PS4 polynucleotides described here, can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. The vector comprising the polynucleotide sequence may be transformed into a suitable host cell. Suitable hosts may include bacterial, yeast, insect and fungal cells.

1510 The term "transformed cell" includes cells that have been transformed by use of recombinant DNA techniques. The transformation typically occurs by insertion of one or more nucleotide sequences into a cell that is to be transformed. The inserted nucleotide sequence may be a heterologous nucleotide sequence (i.e. is a sequence that is not natural to the cell that is to be transformed. In addition, or in the alternative, the inserted
1515 nucleotide sequence may be an homologous nucleotide sequence (i.e. is a sequence that is natural to the cell that is to be transformed) - so that the cell receives one or more extra copies of a nucleotide sequence already present in it.

Thus in a further embodiment, we provide a method of making PS4 variant polypeptides and polynucleotides by introducing a polynucleotide into a replicable vector,

1520 introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell.

EXPRESSION CONSTRUCTS

The PS4 nucleic acid may be operatively linked to transcriptional and translational
1525 regulatory elements active in a host cell of interest. The PS4 nucleic acid may also encode a fusion protein comprising signal sequences such as, for example, those derived from the glucoamylase gene from *Schwanniomyces occidentalis*, α -factor mating type gene from *Saccharomyces cerevisiae* and the TAKA-amylase from *Aspergillus oryzae*. Alternatively, the PS4 nucleic acid may encode a fusion protein comprising a membrane binding
1530 domain.

Expression Vector

The PS4 nucleic acid may be expressed at the desired levels in a host organism using an expression vector.

An expression vector comprising a PS4 nucleic acid can be any vector which is
1535 capable of expressing the gene encoding PS4 nucleic acid in the selected host organism, and the choice of vector will depend on the host cell into which it is to be introduced. Thus, the vector can be an autonomously replicating vector, i.e. a vector that exists as an episomal entity, the replication of which is independent of chromosomal replication, such as, for example, a plasmid, a bacteriophage or an episomal element, a minichromosome or
1540 an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome.

Components of the Expression Vector

The expression vector typically includes the components of a cloning vector, such
1545 as, for example, an element that permits autonomous replication of the vector in the selected host organism and one or more phenotypically detectable markers for selection purposes. The expression vector normally comprises control nucleotide sequences encoding a promoter, operator, ribosome binding site, translation initiation signal and optionally, a repressor gene or one or more activator genes. Additionally, the expression

1550 vector may comprise a sequence coding for an amino acid sequence capable of targeting the PS4 variant polypeptide to a host cell organelle such as a peroxisome or to a particular host cell compartment. Such a targeting sequence includes but is not limited to the sequence SKL. In the present context, the term 'expression signal' includes any of the above control sequences, repressor or activator sequences. For expression under the
1555 direction of control sequences, the nucleic acid sequence the PS4 variant polypeptide is operably linked to the control sequences in proper manner with respect to expression.

Preferably, a polynucleotide in a vector is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" means that the components
1560 described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The control sequences may be modified, for example by the addition of further
1565 transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators. The control sequences may in particular comprise promoters.

Promoter

In the vector, the nucleic acid sequence encoding for the variant PS4 polypeptide is
1570 operably combined with a suitable promoter sequence. The promoter can be any DNA sequence having transcription activity in the host organism of choice and can be derived from genes that are homologous or heterologous to the host organism.

Bacterial Promoters

Examples of suitable promoters for directing the transcription of the modified
1575 nucleotide sequence, such as PS4 nucleic acids, in a bacterial host include the promoter of the *lac* operon of *E. coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis* α -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α -amylase gene (*amyQ*), the promoters of the *Bacillus subtilis*

1580 *xylA* and *xylB* genes and a promoter derived from a *Lactococcus* sp.-derived promoter including the P170 promoter. When the gene encoding the PS4 variant polypeptide is expressed in a bacterial species such as *E. coli*, a suitable promoter can be selected, for example, from a bacteriophage promoter including a T7 promoter and a phage lambda promoter.

1585 *Fungal Promoters*

For transcription in a fungal species, examples of useful promoters are those derived from the genes encoding the, *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral α -amylase, *A. niger* acid stable α -amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline
1590 protease, *Aspergillus oryzae* triose phosphate isomerase or *Aspergillus nidulans* acetamidase.

Yeast Promoters

Examples of suitable promoters for the expression in a yeast species include but are not limited to the Gal 1 and Gal 10 promoters of *Saccharomyces cerevisiae* and the
1595 *Pichia pastoris* AOX1 or AOX2 promoters.

HOST ORGANISMS

(I) Bacterial Host Organisms

Examples of suitable bacterial host organisms are gram positive bacterial species such as *Bacillaceae* including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*,
1600 *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus megaterium* and *Bacillus thuringiensis*, *Streptomyces* species such as *Streptomyces murinus*, lactic acid bacterial species including *Lactococcus* spp. such as *Lactococcus lactis*, *Lactobacillus* spp. including *Lactobacillus reuteri*, *Leuconostoc* spp., *Pediococcus* spp. and *Streptococcus*
1605 spp. Alternatively, strains of a gram-negative bacterial species belonging to *Enterobacteriaceae* including *E. coli*, or to *Pseudomonadaceae* can be selected as the host organism.

(II) Yeast Host Organisms

A suitable yeast host organism can be selected from the biotechnologically relevant yeasts species such as but not limited to yeast species such as *Pichia sp.*, *Hansenula sp* or *Kluyveromyces*, *Yarrowinia* species or a species of *Saccharomyces* including *Saccharomyces cerevisiae* or a species belonging to *Schizosaccharomyce* such as, for example, *S. Pombe* species.

Preferably a strain of the methylotrophic yeast species *Pichia pastoris* is used as the host organism. Preferably the host organism is a *Hansenula* species.

(III) Fungal Host Organisms

Suitable host organisms among filamentous fungi include species of *Aspergillus*, e.g. *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus tubigenensis*, *Aspergillus awamori* or *Aspergillus nidulans*. Alternatively, strains of a *Fusarium* species, e.g. *Fusarium oxysporum* or of a *Rhizomucor* species such as *Rhizomucor miehei* can be used as the host organism. Other suitable strains include *Thermomyces* and *Mucor* species.

Protein Expression and Purification

Host cells comprising polynucleotides may be used to express polypeptides, such as variant PS4 polypeptides, fragments, homologues, variants or derivatives thereof. Host cells may be cultured under suitable conditions which allow expression of the proteins. Expression of the polypeptides may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG.

Polypeptides can be extracted from host cells by a variety of techniques known in the art, including enzymatic, chemical and/or osmotic lysis and physical disruption. Polypeptides may also be produced recombinantly in an *in vitro* cell-free system, such as the TnT™ (Promega) rabbit reticulocyte system.

EXAMPLES

Example 1. Cloning of PS4

Pseudomonas sacharophila is grown overnight on LB media and chromosomal DNA is isolated by standard methods (Sambrook J, 1989). A 2190 bp fragment containing

the PS4 open reading frame (Zhou *et al.*, 1989) is amplified from *P. sacharophila* chromosomal DNA by PCR using the primers P1 and P2 (see Table 3). The resulting
1640 fragment is used as a template in a nested PCR with primers P3 and P4, amplifying the openreading frame of PS4 without its signal sequence and introducing a NcoI site at the 5' end of the gene and a BamHI site at the 3'end. Together with the NcoI site a codon for a N-terminal Methionine is introduced, allowing for intracellular expression of PS4. The 1605 bp fragment is cloned into pCRBLUNT TOPO (Invitrogen) and the integrity of the
1645 construct analysed by sequencing. The *E.coli Bacillus* shuttle vector pDP66K (Penninga *et al.*, 1996) is modified to allow for expression of the PS4 under control of the P32 promoter and the *ctgase* signal sequence. The resulting plasmid, pCSmta is transformed into *B. subtilis*.

A second expression construct is made in which the starch binding domain of PS4
1650 is removed. In a PCR with primers P3 and P6 (Table 3) on pCSmta, a truncated version of the mta gene is generated. The full length mta gene in pCSmta is exchanged with the truncated version which resulted in the plasmid pCSmta-SBD.

Example 2. Site Directed Mutagenesis of PS4

Mutations are introduced into the mta gene by 2 methods. Either by a 2 step PCR
1655 based method, or by a Quick Exchange method (QE). For convenience the mta gene is split up in 3 parts; a PvuI-FspI fragment, a FspI-PstI fragment and a PstI-AspI fragment, further on referred to as fragment 1, 2 and 3 respectively.

In the 2 step PCR based method, mutations are introduced using Pfu DNA
polymerase (Stratagene). A first PCR is carried out with a mutagenesis primer (Table 4)
1660 for the coding strand plus a primer downstream on the lower strand (either 2R or 3R Table 3). The reaction product is used as a primer in a second PCR together with a primer upstream on the coding strand. The product of the last reaction is cloned into pCRBLUNT topo (Invitrogen) and after sequencing the fragment is exchanged with the corresponding fragment in pCSmta.

1665 Using the Quick Exchange method (Stratagene), mutations are introduced using two complementary primers in a PCR on a plasmid containing the mta gene, or part of the mta gene.

For this purpose a convenient set of plasmids is constructed, comprising of 3 SDM plasmids and 3 pCSA plasmids. The SDM plasmids each bear 1 of the fragments of the mta gene as mentioned above, in which the desired mutation is introduced by QE. After verification by sequencing, the fragments are cloned into the corresponding recipient pCSA plasmid. The pCSA plasmids are inactive derivatives from pCSmta. Activity is restored by cloning the corresponding fragment from the SDM plasmid, enabling easy screening.

Table 3. Primers used in cloning the mta gene, and standard primers used in construction of site directed mutants with the 2 step PCR method.

| Primer | Primer sequence | Introduced site |
|--------|---|-----------------|
| P1 | 5'- ATG ACG AGG TCC TTG TTT TTC | |
| P2 | 5'- CGC TAG TCG TCC ATG TCG | |
| P3 | 5'- GCC ATG GAT CAG GCC GGC AAG AGC CCG | NcoI |
| P4 | 5'- TGG ATC CTC AGA ACG AGC CGC TGG T | BamHI |
| P6 | 5'- GAA TTC AGC CGC CGT CAT TCC CGC C | EcoRI |
| 2L | 5'-AGA TTT ACG GCA TGT TTC GC | |
| 2R | 5'-TAG CCG CTA TGG AAG CTG AT | |
| 3L | 5'-TGA CCT TCG TCG ACA ACC AC | |
| 3R | 5'-GAT AGC TGC TGG TGA CGG TC | |

1680

Table 4: Primers used to introduce site directed mutations in *mta*

| Mutation | Oligo Sequence | Modification | Strand | Purpose |
|-------------------|--|--------------|--------|---------|
| G134R | CTGCCGCGCCGCCAGcGCTTCTGGCG | | + | SDM |
| G134R - | cgccagaagcgctggccggccggcag | | - | SDM |
| I157L | GACGGTGACCGCTTCcTgGGCGGCGAGTCG | | + | SDM |
| I151L - | cgactcgccgcccaggaagcggtcacgctc | | - | SDM |
| G223A | GGCGAGCTGTGGAAAgccCCTTCTGAATATCCG | | + | SDM |
| G223A - | cggatattcagaaggggcttccacagctcgcc | | - | SDM |
| H307L | gaacGGCGGCCAGCACctgTGGGCGCTGCAG | | + | SDM |
| H307L - | ctgcagcgccacaggtgctggccgcccgttc | | - | SDM |
| S334P, D343E | GTA CTGGccgCACATGTACGACTGGGGCTACGGC gaaTTCATC | | + | SDM |
| S334P, D343E - | gatgaattcgccgtagccccagtcgtacatgtgcggccagtac | | - | SDM |

Table 5. Features of the SDM and pCSA plasmids

| Plasmid | Features/construction |
|---------|---|
| SDM1 | pBlueSK+ 480 bp Sall-StuI fragment mta |
| SDM2 | pBlueSK+ 572 bp SacII-PstI fragment mta |
| SDM3 | pBlueSK+ 471 bp Sall-StuI fragment mta |
| pCSA1 | FseI site filled in with Klenow ----> frameshift in mta |
| pCSA2 | FspI-PstI fragment of mta replaced with 'junk-DNA' |
| pCSA3 | PstI-AspI fragment of mta replaced with 'junk-DNA' |

1685 Example 3. Multi SDM

The PS4 variants were generated using a QuickChange® Multi Site Directed Mutagenesis Kit (Stratagene) according to the manufactures protocol with some modifications as described.

1690

Step 1: Mutant Strand Synthesis Reaction (PCR)

- Inoculate 3ml. LB (22g/l Lennox L Broth Base, Sigma) + antibiotics (0,05 µg/ml kanamycin, Sigma) in a 10ml Falcon tube
- 1695 - Incubate o/n 37°C, ca. 200 rpm.
- Spin down the cells by centrifugation (5000 rpm/5 min)
- Pour off the medium
- Prepare ds-DNA template using QIAGEN Plasmid Mini Purification Protocol

- 1700 1. The mutant strand synthesis reaction for thermal cycling was prepared as follow:

PCR Mix:

- 2,5 µl 10X QuickChange® Multi reaction buffer
- 0,75 µl QuickSolution
- 1705 X µl Primers $\left(\begin{array}{l} \text{primer length 28-35 bp} \rightarrow 10 \text{ pmol} \\ \text{primer length 24-27 bp} \rightarrow 7 \text{ pmol} \\ \text{primer length 20-23 bp} \rightarrow 5 \text{ pmol} \end{array} \right)$
- 1 µl dNTP mix
- 1710 X µl ds-DNA template (200 ng)
- 1 µl QuickChange® Multi enzyme blend (2,5 U/µl) (*PfuTurbo*® DNA polymerase)
- X µl dH₂O (to a final volume of 25 µl)

- 1715 Mix all components by pipetting and briefly spin down the reaction mixtures.

2. Cycle the reactions using the following parameters:

- 35 cycles of denaturation (96°C/1min)
- primer annealing (62,8°C/1min)
- 1720 elongation (65°C/15min)
- then hold at 4°C

Preheat the lid of the PCR machine to 105°C and the plate to 95°C before the PCR tubes are placed in the machine (ependorf thermal cycler).

1725

Step 2: *Dpn I* Digestion

1. Add 2 μ l *Dpn I* restriction enzyme (10 U/ μ l) to each amplification reaction, mix by pipetting and spin down mixture.
- 1730 2. Incubate at 37°C for ~3 hr.

Step 3: Transformation of XL10-Gold® Ultracompetent Cells

- 1735 1. Thaw XL10-Gold cells on ice. Aliquot 45 μ l cells per mutagenesis reaction to prechilled Falcon tubes.
2. Turn on the waterbath (42°C) and place a tube with NZY⁺ broth in the bath to preheat.
- 1740 3. Add 2 μ l β -mercaptoethanol mix to each tube. Swirl and tap gently and incubate 10 min on ice, swirling every 2 min.
4. Add 1,5 μ l *Dpn I*-treated DNA to each aliquot of cells, swirl to mix and incubate on ice for 30 min.
5. Heat-pulse the tubes in 42°C waterbath for 30 s and place on ice for 2 min.
- 1745 6. Add 0.5 ml preheated NZY⁺ broth to each tube and incubate at 37°C for 1hr with shaking at 225-250 rpm.
7. Plate 200 μ l of each transformation reaction on LB plates (33,6 g/l Lennox L Agar, Sigma) containing 1% starch and 0,05 μ g/ml kanamycin
8. Incubate the transformation plates at 37°C overnight.

1750

Table 6. Primer table for pPD77d14:

| Mutation | Oligo Sequence | Modification | Strand | Purpose |
|-----------------|--|--------------|--------|---------|
| N33Y, D34N | GCGAAGCGCCCTACAAC TGGTACAAC | 5' phosphate | + | MSDM |
| K71R | CCGACGGCGGCAGGTCCGGCG | 5' phosphate | + | MSDM |
| G87S | CAAGAACAGCCGCTACGGCAGCGAC | 5' phosphate | + | MSDM |
| G121D | CACATGAACCGCGACTACCCGGACAAG | 5' phosphate | + | MSDM |
| G134R | CTGCCGGCCGGCCAGcGCTTCTGGCG | 5' phosphate | + | MSDM |
| A141P | CGCAACGACTGCGCCGACCCGGG | 5' phosphate | + | MSDM |
| I157L | GACGGTGACCGCTTCcTgGGCGGCGAGTCG | 5' phosphate | + | MSDM |
| L178F, A179T | CGCGACGAGTTTACCAACCTGCG | 5' phosphate | + | MSDM |
| G223A | GGCGAGCTGTGGAAAgccCCTTCTGAATATCCG | 5' phosphate | + | MSDM |
| H307L | gaacGGCGGCCAGCACctgTGGGCGCTGCAG | 5' phosphate | + | MSDM |
| S334P, D343E | GTA CTGGccgCACATGTACGACTGGGGCTACGGC gaaTTCATC | 5' phosphate | + | MSDM |

1755 Table 7. Primer table for pPD77d20:

| Mutation | Oligo Sequence | Modification | Strand | Purpose |
|-----------------|--|--------------|--------|---------|
| N33Y, D34N | GCGAAGCGCCCTACAAC TGGTACAAC | 5' phosphate | + | MSDM |
| K71R | CCGACGGCGGCAGGTCCGGCG | 5' phosphate | + | MSDM |
| G121D | CACATGAACCGCGACTACCCGGACAAG | 5' phosphate | + | MSDM |
| G134R | CTGCCGGCCGGCCAGcGCTTCTGGCG | 5' phosphate | + | MSDM |
| A141P | CGCAACGACTGCGCCGACCCGGG | 5' phosphate | + | MSDM |
| I157L | GACGGTGACCGCTTCcTgGGCGGCGAGTCG | 5' phosphate | + | MSDM |
| L178F, A179T | CGCGACGAGTTTACCAACCTGCG | 5' phosphate | + | MSDM |
| G223A | GGCGAGCTGTGGAAAgccCCTTCTGAATATCCG | 5' phosphate | + | MSDM |
| H307L | gaacGGCGGCCAGCACctgTGGGCGCTGCAG | 5' phosphate | + | MSDM |
| S334P, D343E | GTA CTGGccgCACATGTACGACTGGGGCTACGGC gaaTTCATC | 5' phosphate | + | MSDM |

Table 8. Primer table for pPD77d34 (pSac-D34)

| Mutation | Oligo Sequence | Modification | Strand | Purpose |
|-----------------|-------------------------------------|--------------|--------|---------|
| N33Y, D34N | GCGAAGCGCCCTACAAC TGGTACAAC | 5' phosphate | + | MSDM |
| G121D | CACATGAACCGCGACTACCCGGACAAG | 5' phosphate | + | MSDM |
| G134R | CTGCCGGCCGGCCAGcGCTTCTGGCG | 5' phosphate | + | MSDM |
| A141P | CGCAACGACTGCGCCGACCCGGG | 5' phosphate | + | MSDM |
| I157L | GACGGTGACCGCTTCcTgGGCGGCGAGTCG | 5' phosphate | + | MSDM |
| L178F, A179T | CGCGACGAGTTTACCAACCTGCG | 5' phosphate | + | MSDM |
| G223A | GGCGAGCTGTGGAAAgccCCTTCTGAATATCCG | 5' phosphate | + | MSDM |
| H307L | gaacGGCGGCCAGCACctgTGGGCGCTGCAG | 5' phosphate | + | MSDM |
| S334P | GTA CTGGccgCACATGTACGACTGGGGCTACGGC | 5' phosphate | + | MSDM |

1760

Vector system based on pPD77

The vector system used for pPD77 is based on pCRbluntTOPOII (invitrogen). The zeocin resistance cassette has been removed by pmlI, 393 bp fragment removed. The expression cassette from the pCC vector (P32-ssCGTase-PS4-tt) has then been inserted into the vector.

Ligation of PS4 variant into pCCMini

1770 The plasmid which contain the relevant mutations (created by MSDM) is cut with restriction enzyme Nco I and Hind III (Biolabs):

3 μ g plasmid DNA, X μ l 10x buffer 2, 10 units NcoI, 20 units HindIII.

Incubation 2h at 37°C

1775 Run digestion on a 1% agarose gel. Fragments sized 1293 bp (PS4 gene) is cut out of the gel and purified using Qiagen gel purification kit.

1780 The vector pCCMini is then cut with restriction enzymes, Nco I and Hind III, and the digestion is then run on a 1% agarose gel. The fragment sized 3569 bp is cut out of the gel and purified using Qiagen gel purification kit.

Ligation: Use Rapid DNA ligation kit (Roche)
Use the double amount of insert compared to vector

1785 e.g. 2 µl insert (PS4 gene)
1 µl vector
5 µl T4 DNA ligation buffer 2xconc
1 µl dH₂O
1 µl T4 DNA ligase

1790 Ligate 5 min/RT

Transform the ligation into One Shot TOPO competent cells according to manufactures protocol (Invitrogen). Use 5 μ l ligation pr. transformation.

1795 Plate 50 µl transformation mix onto LB plates (33,6 g/l Lennox L Agar, Sigma)
containing 1% starch and 0,05 µg/ml kanamycin. Vectors containing insert (PS4 variants)
can be recognised by halo formation on the starch plates.

Example 4. Transformation into *Bacillus subtilis* (Protoplast Transformation)

Bacillus subtilis (strain DB104A; Smith et al. 1988; Gene 70, 351-361) is transformed with the mutated pCS-plasmids according to the following protocol.

A. Media for protoplasting and transformation

2 x SMM per litre: 342 g sucrose (1 M); 4.72 g sodium maleate (0.04 M); 8:12 g $\text{MgCl}_{2} \cdot 6\text{H}_2\text{O}$ (0.04 M); pH 6.5 with concentrated

- 1805 NaOH. Distribute in 50-ml portions and autoclave for 10 min.
- 1810 4 x YT (1/2 NaCl) 2 g Yeast extract + 3.2 g Tryptone + 0.5 g NaCl per 100 ml.
SMMF mix equal volumes of 2 x SMM and 4 x YT.
PEG 10 g polyethyleneglycol 6000 (BDH) or 8000 (Sigma) in 25 ml 1 x SMM (autoclave for 10 min.).

B. Media for plating/regeneration

- 1815 agar 4% Difco minimal agar. Autoclave for 15 min.
- sodium succinate 270 g/l (1 M), pH 7.3 with HCl. Autoclave for 15 min.
- 1820 phosphate buffer 3.5 g K_2HPO_4 + 1.5 g KH_2PO_4 per 100ml. Autoclave for 15 min.
- MgCl₂ 20.3 g MgCl₂, 6H₂O per 100 ml (1 M).
casamino acids 5% (w/v) solution. Autoclave for 15 min.
yeast extract 10 g per 100 ml, autoclave for 15 min.
1825 glucose 20% (w/v) solution. Autoclave for 10 min.

DM3 regeneration medium: mix at 60 C (waterbath; 500-ml bottle):

- 1830 250 ml sodium succinate
50 ml casamino acids
25 ml yeast extract
50 ml phosphate buffer
15 ml glucose
10 ml MgCl₂
1835 100 ml molten agar

Add appropriate antibiotics: chloramphenicol and tetracycline, 5 ug/ml; erythromycin, 1 ug/ml. Selection on kanamycin is problematic in DM3 medium: concentrations of 250 ug/ml may be required.

1840

C. Preparation of protoplasts

1. Use detergent-free plastic or glassware throughout.
 2. Inoculate 10 ml of 2 x YT medium in a 100-ml flask from a single colony.
- 1845 Grow an overnight culture at 25-30 C in a shaker (200 rev/min).
3. Dilute the overnight culture 20 fold into 100 ml of fresh 2 x YT medium (250-ml flask) and grow until OD₆₀₀ = 0.4-0.5 (approx. 2h) at 37C in a shaker (200-250 rev/min).

4. Harvest the cells by centrifugation (9000g, 20 min, 4 C).
- 1850 5. Remove the supernatant with pipette and resuspend the cells in 5 ml of SMMP + 5 mg lysozyme, sterile filtered.
6. Incubate at 37 C in a waterbath shaker (100 rev/min).

After 30 min and thereafter at 15 min intervals, examine 25 ul samples by microscopy. Continue incubation until 99% of the cells are protoplasted (globular appearance). Harvest the protoplasts by centrifugation (4000g, 20 min, RT) and pipet off the supernatant. Resuspend the pellet gently in 1-2 ml of SMMP.

The protoplasts are now ready for use. (Portions (e.g. 0.15 ml) can be frozen at -80 C for future use (glycerol addition is not required). Although this may result in some reduction of transformability, 10⁶ transformants per ug of DNA can be obtained with frozen protoplasts).

D. Transformation

1. Transfer 450 ul of PEG to a microtube.
2. Mix 1-10 ul of DNA (0.2 ug) with 150 ul of protoplasts and add the mixture to the microtube with PEG. Mix immediately, but gently.
- 1865 3. Leave for 2 min at RT, and then add 1.5 ml of SMMP and mix.
4. Harvest protoplasts by microfuging (10 min, 13.000 rev/min (10-12.000 g)) and pour off the supernatant. Remove the remaining droplets with a tissue.

Add 300 ul of SMMP (do not vortex) and incubate for 60-90 min at 37 C in a waterbath shaker (100 rev/min) to allow for expression of antibiotic resistance markers.

1870 (The protoplasts become sufficiently resuspended through the shaking action of the waterbath.). Make appropriate dilutions in 1 x SSM and plate 0.1 ml on DM3 plates

Example 5. Fermentation of PS4 Variants in Shake Flasks

The shake flask substrate is prepared as follows:

| Ingredient | %(w/v) |
|-----------------------|--------|
| Water | - |
| Yeast extract | 2 |
| Soy Flour | 2 |
| NaCl | 0.5 |
| Dipotassium phosphate | 0.5 |
| Antifoam agent | 0.05 |

The substrate is adjusted to pH 6.8 with 4N sulfuric acid or sodium hydroxide before autoclaving. 100 ml of substrate is placed in a 500 ml flask with one baffle and autoclaved for 30 minutes. Subsequently, 6 ml of sterile dextrose syrup is added. The dextrose syrup is prepared by mixing one volume of 50% w/v dextrose with one volume of water followed by autoclaving for 20 minutes.

The shake flask are inoculated with the variants and incubated for 24 hours at 35°C/180rpm in an incubator. After incubation cells are separate from broth by centrifugation (10.000 x g in 10 minutes) and finally, the supernatant is made cell free by microfiltration at 0,2µm. The cell free supernatant is used for assays and application tests.

Example 6. Amylase Assays

Betamyl assay

One Betamyl unit is defined as activity degrading 0,0351 mmole per 1 min. of PNP-coupled maltopentaose so that 0,0351 mmole PNP per 1 min. can be released by excess α-glucosidase in the assay mix. The assay mix contains 50 ul 50 mM Na-citrate, 5 mM CaCl₂, pH 6,5 with 25 ul enzyme sample and 25 ul Betamyl substrate (Glc5-PNP and α-glucosidase) from Megazyme, Ireland (1 vial dissolved in 10 ml water). The assay mix is incubated for 30 min. at 40C and then stopped by adding 150 ul 4% Tris. Absorbance at 420 nm is measured using an ELISA-reader and the Betamyl activity is calculate based on Activity = A₄₂₀ * d in Betamyl units/ml of enzyme sample assayed.

Endo-amylase assay

The endo-amylase assay is identical to the Phadebas assay run according to manufacturer (Pharmacia & Upjohn Diagnostics AB).

Exo-specificity

The ratio of exo-amylase activity to Phadebas activity was used to evaluate exo-specificity.

Specific activity

1900 For the PSac-D14, PSac-D20 and PSac-D34 variants we find an average specific activity of 10 Betamyl units per microgram of purified protein measured according to Bradford (1976; Anal. Biochem. 72, 248). This specific activity is used for based on activity to calculate the dosages used in the application trials.

Example 7. Half-life Determination

1905 $t_{1/2}$ is defined as the time (in minutes) during which half the enzyme activity is inactivated under defined heat conditions. In order to determine the half life of the enzyme, the sample is heated for 1-10 minutes at constant temperatures of 60°C to 90°C. The half life is calculated based on the residual Betamyl assay.

Procedure: In an Eppendorf vial, 1000 μ l buffer is preheated for at least 10 minutes at 60°C or higher. The heat treatment of the sample is started addition of 100 μ l of the sample to the preheated buffer under continuous mixing (800 rpm) of the Eppendorf vial in an heat incubator (Termomixer comfort from Eppendorf). After 0, 2, 4, 6, 8 and 9 minutes of incubation, the treatment is stopped by transferring 45 μ l of the sample to 1000 μ l of the buffer equilibrated at 20°C and incubating for one minute at 1500 rpm and at 1915 20°C. The residual activity is measured with the Betamyl assay.

Calculation: Calculation of $t_{1/2}$ is based on the slope of \log_{10} (the base-10 logarithm) of the residual Betamyl activity versus the incubation time. $t_{1/2}$ is calculated as $\text{Slope}/0.301=t_{1/2}$.

Example 8. Results1920 **Table 9. Biochemical properties of PSac-variants compared to wild-type PSac-cc1**

| Variant | t _{1/2} -75 | t _{1/2} -80 | Betamyl/ Phadeba s | Mutations |
|----------------------------|----------------------|----------------------|--------------------------|--|
| PSac-cc1 | <0,5 | | 40 | |
| PSac-D3 | 9.3 | 3 | 43 | N33Y, D34N, K71R, G134R, A141P, I157L, L178F, A179T, G223A, H307L, D343E, S334P |
| PSac-D14 (SEQ ID NO: 4) | 9.3 | 2.7 | 65 | N33Y, D34N, K71R, G87S, G121D, G134R, A141P, I157L, L178F, A179T, G223A, H307L, D343E, S334P |
| PSac-D20 (SEQ ID NO: 3) | 7.1 | 2.7 | 86 | N33Y, D34N, K71R, G121D, G134R, A141P, I157L, L178F, A179T, G223A, H307L, D343E, S334P |
| PSac-D34 (SEQ ID NO: 2) | 8.4 | 2.9 | 67 | N33Y, D34N, G121D, G134R, A141P, I157L, L178F, A179T, G223A, H307L, S334P |
| PSac-pPD77d10 | 3.7 | | 61 | G121D, G134R, A141P, I157L, G223A, H307L, S334P, D343E |
| PSac-pPD77d32 | | 2.5 | 52 | G134R, A141P, I157L, G223A, H307L, S334P, L178F+A179T |
| PSac-pPD77d33 | 7.1 | 3 | 51 | N33Y, D34N, G134R, A141P, I157L, L178F, A179T, G223A, H307L, S334P |
| PSac-pPD77d36 | | 2.8 | 77 | G87S, G121D, G134R, A141P, I157L, G223A, H307L, S334P, K71R, L178F, A179T |
| PSac-pPD77d38 | 7.9 | 2.5 | 77 | G121D, G134R, A141P, I157L, G223A, H307L, S334P, L178F+A179T |
| PSac-pPD77d40 | 10.26 | 3.1 | 63 | N33Y D34N K71R G121D G134R A141P I157L L178F+A179T G223A H307L S334P |

Experiments are done with versions of each of the variants listed in the table above, but without mutations are position 33 (i.e., having wild type residue N at this position). Similar results are obtained for such versions, as for those with mutation N33Y.

1925 Experiments are also done with versions of each of the variants listed in the table above, but without mutations are position 34 (i.e., having wild type residue D at this position). Similar results are obtained for such versions, as for those with mutation D34N.

Example 9. Model System Baking Tests

The doughs are made in the Farinograph at 30.0°C. 10.00 g reformed flour is weighed out and added in the Farinograph; after 1 min. mixing the reference/sample
1930 (reference = buffer or water, sample = enzyme+ buffer or water) is added with a sterile

pipette through the holes of the kneading vat. After 30 sec. the flour is scraped off the edges – also through the holes of the kneading vat. The sample is kneaded for 7 min.

1935 A test with buffer or water is performed on the Farinograph before the final reference is run. FU should be 400 on the reference, if it is not, this should be adjusted with, for example, the quantity of liquid. The reference/sample is removed with a spatula and placed in the hand (with a disposable glove on it), before it is filled into small glass tubes (of approx. 4.5 cm's length) that are put in NMR tubes and corked up. 7 tubes per dough are made.

1940 When all the samples have been prepared, the tubes are placed in a (programmable) water bath at 33°C (without corks) for 25 min. and hereafter the water bath is set to stay for 5 min. at 33°C, then to heated to 98°C over 56 min. (1.1°C per minute) and finally to stay for 5 min. at 96°C.

1945 The tubes are stored at 20.0°C in a thermo cupboard. The solid content of the crumb was measured by proton NMR using a Bruker NMS 120 Minispec NMR analyser at day 1, 3 and 7 as shown for crumb samples prepared with 0, 0.5, 1 and 2 ppm PSacD34 in Fig. 2. The lower increase in solid content over time represents the reduction in amylopectin retrogradation. After 7 days of storage at 20.0°C in a thermo cupboard 10-20 mg samples of crumb weighed out and placed in 40 µl aluminium standard DSC capsules and kept at 20°C.

1950 The capsules are used for Differential Scanning Calorimetry on a Mettler Toledo DSC 820 instrument. As parameters are used a heating cycle of 20-95°C with 10°C per min. heating and Gas/flow: N₂/80 ml per min. The results are analysed and the enthalpy for melting of retrograded amylopectin is calculated in J/g.

Example 10. Antistaling Effects

1955 Model bread crumbs are prepared and measured according to Example 8. As shown in Table 2, PS4 variants show a strong reduction of the amylopectin retrogradation after baking as measured by Differential Scanning Calorimetry in comparison to the control. The PS4 variants shows a clear dosage effect.

Example 11. Firmness Effects in Baking Trials

1960 Baking trials were carried out with a standard white bread sponge and dough recipe for US toast. The sponge dough is prepared from 1600 g of flour "All Purpose Classic" from Sisco Mills, USA", 950 g of water, 40 g of soy bean oil and 32 g of dry yeast. The sponge is mixed for 1 min. at low speed and subsequently 3 min. at speed 2 on a Hobart spiral mixer. The sponge is subsequently fermented for 2,5 hours at 35°C, 85% RH
1965 followed by 0,5 hour at 5°C.

Thereafter 400 g of flour, 4 g of dry yeast, 40 g of salt, 2,4 g of calcium propionate, 240 g of high fructose corn sirup (Isosweet), 5 g of the emulsifier PANODAN 205, 5 g of enzyme active soy flour, 30 g of non-active soy flour, 220 g of water and 30 g of a solution of ascorbic acid (prepared from 4 g ascorbic acid solubilised in 500 g of water)
1970 are added to the sponge. The resulting dough is mixed for 1 min. at low speed and then 6 min. on speed 2 on a Diosna mixer. Thereafter the dough is rested for 5 min. at ambient temperature, and then 550 g dough pieces are scaled, rested for 5 min. and then sheeted on Glimek sheeter with the settings 1:4, 2:4, 3:15, 4:12 and 10 on each side and transferred to a baking form. After 60 min. proofing at 43°C at 90% RH the doughs are baked for 29
1975 min. at 218°C

Firmness and resilience were measured with a TA-XT 2 texture analyser. The Softness, cohesiveness and resilience is determined by analysing bread slices by Texture Profile Analysis using a Texture Analyser From Stable Micro Systems, UK. The following settings were used:

1980 Pre Test Speed: 2 mm/s
Test Speed: 2 mm/s
Post Test Speed: 10 mm/s
Rupture Test Distance: 1%
Distance: 40%
1985 Force: 0.098 N
Time: 5.00 sec
Count: 5
Load Cell: 5 kg
Trigger Type: Auto – 0.01 N

1990 Results are shown in Figures 3 and 4.

Example 12. Control of Volume of Danish Rolls

Danish Rolls are prepared from a dough based on 2000 g Danish reform flour (from Cerealia), 120 g compressed yeast, 32 g salt, and 32 g sucrose. Water is added to the dough according to prior water optimisation.

1995 The dough is mixed on a Diosna mixer (2 min. at low speed and 5 min. at high speed). The dough temperature after mixing is kept at 26°C. 1350 g dough is scaled and rested for 10 min. in a heating cabinet at 30°C. The rolls are moulded on a Fortuna molder and proofed for 45 min. at 34°C and at 85% relative humidity. Subsequently the rolls are baked in a Bago 2 oven for 18 min. at 250°C with steam in the first 13 seconds. After
2000 baking the rolls are cooled for 25 min. before weighing and measuring of volume.

The rolls are evaluated regarding crust appearance, crumb homogeneity, capping of the crust, ausbund and specific volume (measuring the volume with the rape seed displacement method).

Based on these criteria it is found that the PS4 variants increase the specific
2005 volume and improve the quality parameters of Danish rolls. Thus PS4 variants are able to control the volume of baked products.

Example 13. Chemically Leavened Vanilla Cake Donut

Vanilla cake donuts are prepared using a standard recipe as follows.

| Ingredients | Grams |
|---|-------|
| <i>Step 1</i> | |
| U.S. Soft wheat Flour (8.8 – 9.0% Protein) | 3600 |
| U.S. Hard Winter Wheat Flour (10.3 – 11.0%) | 2400 |
| Granulated Sugar | 2512 |
| Dried Egg Yolk | 200 |
| Defatted Soy Flour | 370 |
| Nonfat Dried Milk | 200 |
| Soda (USP #2) | 83 |
| Sodium Acid Pyrophosphate #40 | 83 |
| Sodium Acid Pyrophosphate #28 | 36 |
| Salt | 90 |
| Artificial Vanilla Flavor G28395 (DANISCO, New Century, Kansas) | 15 |
| Modified Food Starch (StabiTex Instant 12626, Cerestar, USA, | 30 |

| | |
|--|---------------|
| Hammond, IN) | |
| Cellulose Gum | 10 |
| DIMODAN® PH300 K A Softener (DANISCO, New Century, Kansas) | 41 |
| | |
| <i>Step 2</i> | |
| Soybean Oil | 300 |
| Fluid Lecithin | 30 |
| Total | 10,000 |

2010

Mix Preparation Procedure

1. Use a Hobart A-200 mixer with a paddle and a 20-quart bowl. Combine Step 1 ingredients and blend for 10 minutes on Speed 1. 2. Add Step 2 ingredients over 2 minutes in Speed 1, then blend 18 additional minutes. 3. Run mix through cake finisher to smooth and remove lumps.

2015

Batter Preparation Procedure

1. Use a Hobart A-200 mixer with a paddle and a 12-quart bowl. Mix: 2000 grams; Water: 900 grams. Add water to the bottom of the bowl. 2. Add mix on top. Mix 1 minute on Speed 1, then 2 minutes on Speed 2. 3. The target batter temperature should be 72°F for donuts made in an open kettle fryer.

2020

Frying Procedure

Set fryer temperature for 375° F using well conditioned donut shortening to fry donuts (do not use oil or all-purpose shortening).

For an Open Kettle Fryer

2025

Target a weight of 43 grams per donut using a 1 ¾ inch diameter cutter. Fry for 50 seconds on the first side, flip, then fry 50 - 60 seconds more. Remove from fryer and allow grease to drain and donuts to cool.

Example 14. Fresh keeping effects in Vanilla Cake Donut

Addition of PSac-D34 to Vanilla Cake Donuts (Example 13) improves the fresh keeping as evaluated on day 8 after baking:

2030

| | Hardness | Cohesiveness | Resilience | Freshness | Gumminess | Overall liking |
|------------------|----------|--------------|------------|-----------|-----------|----------------|
| Control | 330 | 0,581 | 0,272 | 5,04 | 4,19 | 4,54 |
| 2 mg/kg PSac-D34 | 240 | 0,646 | 0,295 | 5,53 | 5,46 | 5,15 |

Table 10. Freshness parameters evaluated in Donuts without and with PSac-D34

Hardness, Cohesiveness and Resilience are measured on TPA as described in Example 11.

2035 Freshness, Gumminess and Overall liking are scored by sensory evaluation on a scale from 1 – 9 where 1 is inferior and 9 is best.

It is observed that all quality parameters are improved due to addition of PSac-D34; hardness is reduced, cohesiveness and resilience are increased and freshness, gumminess and overall liking based on sensory evaluation are improved.

REFERENCES

2040 Penninga,D., van der Veen,B.A., Knegt,R.M., van Hijum,S.A., Rozeboom,H.J., Kalk,K.H., Dijkstra,B.W., Dijkhuizen,L. (1996). The raw starch binding domain of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251. *J.Biol.Chem.* 271, 32777-32784.

2045 Sambrook J,F.E.M.T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory, Cold Spring Harbor NY.

Zhou,J.H., Baba,T., Takano,T., Kobayashi,S., Arai,Y. (1989). Nucleotide sequence of the maltotetraohydrolase gene from *Pseudomonas saccharophila*. *FEBS Lett.* 255, 37-41.

2050 Each of the applications and patents mentioned in this document, and each document cited or referenced in each of the above applications and patents, including during the prosecution of each of the applications and patents (“application cited documents”) and any manufacturer’s instructions or catalogues for any products cited or mentioned in each of the applications and patents and in any of the application cited documents, are hereby incorporated herein by reference. Furthermore, all documents cited
2055 in this text, and all documents cited or referenced in documents cited in this text, and any manufacturer’s instructions or catalogues for any products cited or mentioned in this text, are hereby incorporated herein by reference.

2060 Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed

should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the claims.

2065

SEQUENCE LISTINGS**SEQ ID NO: 1**

PS4 reference sequence, derived from *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence.

2070 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APNDWYNILR QQASTIAADG FSAIWMPVPW
 61 RDFSSWTDGG KSGGGEYFW HDFNKNGRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 121 GYPDKKEINLP AGQGFWRNDC ADPGNYPNDC DDGDRFIGGE SDLNTGHPQI YGMFRDELAN
 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKGPSEYPSW DWRNTASWQQ
 241 IIKDWSRAK CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 2075 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWSHMYDWG YGDFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 421 GDGGGNDGGE GGLVNVNFRD DNGVTQMGDS VYAVGNVSQL GNWSPASAVR LTDTSSYPTW
 481 KGSIALPDGQ NVEWKCLIRN EADATLVRQW QSGGNNQVQA AAGASTSGSF

2080 SEQ ID NO: 2

PSac-D34 sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 11 substitutions and deletion of the starch binding domain.

 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG FSAIWMPVPW
 61 RDFSSWTDPG KSGGGEYFW HDFNKNGRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 2085 121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSRAK CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 2090 421 GDGGGNDGG

SEQ ID NO: 3

PSac-D20 sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 13 substitutions and deletion of the starch binding domain.

2095 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG FSAIWMPVPW
 61 RDFSSWTDPG RSGGGEYFW HDFNKNGRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSRAK CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 2100 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 421 GDGGGNDGG

SEQ ID NO: 4

2105 PSac-D14 sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 14 substitutions and deletion of the starch binding domain.

 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG FSAIWMPVPW

2110 61 RDFSSWTDPG RSGGGEGYFW HDFNKNRSRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEI WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSRAK CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSFG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGEFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 421 GDGGGNDGG

2115 SEQ ID NO: 5

Pseudomonas saccharophila Glucan 1,4-alpha-maltotetrahydrolase precursor (EC 3.2.1.60) (G4-amylase) (Maltotetraose-forming amylase) (Exo-maltotetraohydrolase) (Maltotetraose-forming exo-amylase). SWISS-PROT accession number P22963.

2120 MSHILRAAVL AAVLLPFPAL ADQAGKSPAG VRYHGGDEII LQGFHWNVVR EAPNDWYNIL
 RQASTIAAD GFSAIWMPVP WRDFSSWTDG GKSGGGEGYF WHDFNKNRGY GSDAQLRQAA
 GALGGAGVKV LYDVVPNHMN RGYDPKEINL PAGQGFWRND CADPGNYPND CDDGDRFIGG
 ESDLNTGHPQ IYGMFRDELA NLRSGYGAGG FRFDFVRGYA PERVDSWMSD SADSSFCVGE
 LWKGPSEYPS WDWNTASWQ QIIKDWSRA KCPVDFDFAK ERMQNGSVAD WKHGLNGNPDP
 PRWREVAVTF VDNHDTGYSF GQNGGQHHWA LQDGLIRQAY AYILTSPGTP VVYWSHMYDW
 2125 GYGFIRQLI QVRRTAGVRA DSAISFHSGY SGLVATVSGS QQTLVVALNS DLANPGQVAS
 GSFSEAVNAS NGQVRVWRSG SGDGGGNDGG EGGLVNVNFR CDNGVTQMGD SVYAVGNVSQ
 LGNWSPASAV RLTDTSYPT WKGSIALPDG QNVEWKCLIR NEADATLVRQ WQSGGNNQVQ
 AAAGASTSGS F

2130 SEQ ID NO: 6

P. saccharophila mta gene encoding maltotetraohydrolase (EC number = 3.2.1.60).
 GenBank accession number X16732.

2135 gatcggcgta ggttttcgcat tcgttgccca ggcgatattt cgccggtgcg ccagcagcct
 ggaagcaggc ctggtcgccg ccgcccggcc tggcgccgac gcccgaacgc agatagccgt
 ggaaatcgac cgccagggcc ggcccgccga ccagcagggc ggcaagcagg caggcgggtt
 ttaggacgaa caggggggtgc gcggtgtgct tcatgacgag gtccttggtt ttcttgtaa
 tgcgaatcg atcacgcctt cgtgctgtgt cgcagggcgc agctcggtgg cgaaagcctc
 ggggatggct ccgctggcgg catcctccc accagagatt tcgctggcgc agctcgaggg
 2140 cgtaatcagg atgagtgcgg cgtaatccct ggggtggggc tacgcccggc agggcgaga
 tgattgccag gggccttcgg cctggccact acgcccctg caactgggag ggggaggttg
 gtggtcgggg cgtgcagggg cagcctgcgg gtgcccgtcg aagaccggc cggcggtcat
 cctcgtccgg cggccttgcc gtaggatacc cgaacaagca caagaaccgg agtattgcga
 tgagccacat cctgcgtgcc gccgtatttg cggcggtcct gctgccgttt ccgcactgg
 ccgatcaggc cggcaagagc ccggccgggg tgcgctacca cggcggcgac gaaatcatcc
 2145 tccagggctt cactggaac gtgctccgag aagcgcacca cgactggtac aacatcctcc
 gccaacaggc ctcgacgatc gcggccgacg gcttctcggc aatctggatg ccggtgcctt
 ggcgtgactt ctccagctgg accgacggcg gcaagtcggg cggcggcgaa ggctacttct
 ggcagcactt caacaagaac ggccgctacg gcagcgacgc ccagctgcgc caggccggcg
 2150 gcgcactcgg tggcgccggg gtgaaggtgc tctacgatgt ggtgcccatt cacatgaacc
 gcggctaccc ggacaaggag atcaacctgc cggccggcca gggcttctgg gcacagact
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 ccgagcgggt cgacagctgg atgagcgaca gcgccgacag cagcttctgc gttggcgagc
 2155 tgtggaaagg cccttctgaa tatccgagct gggactggcg caacacggcg agctggcagc

2160 agatcatcaa ggactggtcc gaccggggcca agtgcccggt gttcgacttc gctctcaagg
 agcgcgatgca gaacgggtcg gtcgcccact ggaagcatgg cctcaatggc aaccccgacc
 cgcgctggcg cgaggtggcg gtgaccttcg tcgacaacca cgacaccggc tattcgccccg
 ggcagaacgg cgccagcac cactggggcg tgcaggacgg gctgatccgc caggcctacg
 cctacatcct caccagcccg ggcacgcccg tgggtgtactg gtcgcacatg tacgactggg
 gctacggcga cttcatccgc cagctgatcc aggtgcccgc caccgcccgc gtgcgcgcgc
 attcggcgat cagcttccat agcggctaca gcggtctggg cgtaccgtc agcggcagcc
 agcagaccct ggtgggtggcg ctcaactccg atctggccaa ccccgccag gttgccagcg
 gcagcttcag cgagggcggtc aacgccagca acggccagg ggcggtctgg cgcagcggta
 2165 gcggcgatgg cggcgggaat gacggcgcg aggggtggctt ggtcaatgtg aactttcgct
 gcgacaacgg cgtgacgcag atgggcgaca gcgtctacgc ggtgggcaac gtcagccagc
 tcggcaactg gagcccgcc tccgcggtac ggctgaccga caccagcagc tatccgacct
 ggaagggcag catcgccctg cctgacgggtc agaacgtgga atggaagtgc ctgatccgca
 acgagggcga cgcgacgctg gtgcgtcagt ggcaatcggg cggcaacaac cagggtccagg
 2170 ccgcccggcg cgcgagcacc agcggctcgt tctgacgaca tgcccggccc gctcggcta
 cgctacgcc gggcggtccc tcccgaacca ggggtgggcag ggaggaggcc ggcgacgggc
 cgggcccgcg atgctggcac gacaaccata aaagccttcg cgtgcgctg tcgtatcagg
 agctgttcat gttggcccag acccgctcga cccctttccg gcttggcttc ctggcccggc
 tgtacctgct gatcgccgca ctgggtggct tgcgtgatgt ggtagccggc accagcctgg
 2175 ttgccatcgg ccgctgcaa ggcaatgcc agcaaatctc gtcgaccgcg tcgctctgc
 tggtcagcga gagcttcttc ggtacgttgc agagcctgac gcagaacctg tccgacgccc
 tgcccgagga ccggcctgac cagctcgacg gctatgtcgg ccggcatcgc acgctgcagg
 accagggcct cgagctgttc gcccagctgg agcgggtgac gccggcacat gccgagacca
 agcaagcctg ggcggcgtgt tgccggagct cgaccgccc agcctggcgc tgatcgatgc
 2180 gcacgcgacc tgctcgcgcg tggggcgcaa cgccgtcgcc tgccgcatct gcagctgcag
 ttctcgcggc tcaagcagga cctgctgcag gcgcagttcg tgacgggcga cgagctggtc
 gctattcca tcaagcagtt catcatcccg ctcgagcagg tcgagcgctg ctgttcgatg
 ccatcgcgct gtcttcgac aaggcactcg atgaagcggg tgcgcagatc

2185 SEQ ID NO: 7

PS4 reference sequence, derived from *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence.

1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APNDWYNILR QQAATIAADG FSAIWMPVFW
 61 RDFSSWSDGS KSGGGEGYFW HDFNKNRGY SDAQLRQAAS ALGGAGVKVL YDVPNHNMR
 2190 121 GYPDKEINLP AGQGFWRNDC ADPGNYPNDC DDGDRFIGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RPDFVRGYAP ERVNSWMTDS ADNSFCVGEL WKGPSEYPNW DWRNTASWQQ
 241 IIKDWSDRK CPVDFDFAKE RMQNGSIADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHFWAL QDGLIRQAYA YILTSPGTPV VYWSHMYDWG YGDFIRQLIQ VRRAGVRAD
 361 SAISFHSYGS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSQT
 2195 421 GSGGGEPGAL VSVSFRCDNG ATQMGSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

SEQ ID NO: 8

PS4 reference sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 9 substitutions.

2200

1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQAATIAADG FSAIWMPVFW
 61 RDFSSWSDPS KSGGGEGYFW HDFNKNRGY SDAQLRQAAS ALGGAGVKVL YDVPNHNMR
 121 DYPDKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN

2205 181 LRSQYGAGGF RFDFVRGYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 241 IIKDWSDRK CPVDFDFAKE RMQNGSIADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ VVRAAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSQT
 421 GSGGGEPPAL VSVSFRCDNG ATQMGSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

2210

SEQ ID NO: 9

PStu-D20 sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence
 with 11 substitutions.

2215 1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQAATIAADG FSAIWMPVPW
 61 RDFSSWSDPS RSGGGEYFW HGFNKNGRYG SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
 121 DYPDKINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRGYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 241 IIKDWSDRK CPVDFDFAKE RMQNGSIADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ VVRAAGVRAD
 2220 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSQT
 421 GSGGGEPPAL VSVSFRCDNG ATQMGSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

SEQ ID NO: 10

2225 PStu-D14 sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence
 with 12 substitutions.

2230 1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQAATIAADG FSAIWMPVPW
 61 RDFSSWSDPS RSGGGEYFW HGFNKNSRYG SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
 121 DYPDKINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRGYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 241 IIKDWSDRK CPVDFDFAKE RMQNGSIADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ VVRAAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSQT
 421 GSGGGEPPAL VSVSFRCDNG ATQMGSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 2235 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

SEQ ID NO: 11

Pseudomonas stutzeri (*Pseudomonas perfectomarina*). Glucan 1,4- α -
 maltotetrahydrolase precursor (EC 3.2.1.60) (G4-amylase) (Maltotetraose-forming amylase)
 2240 (Exo-maltotetrahydrolase)(Maltotetraose-forming exo-amylase). SWISS-PROT accession
 number P13507.

2245 MSHILRAAVL AAMLLPLPSM ADQAGKSPNA VRYHGGDEII LQGFHWNVVR EAPNDWYNIL
 RQQAATIAAD GFSAIWMPVP WRDFSSWSDG SKSGGGEYF WHGFNKNGRY GSDAQLRQA
 SALGGAGVKV LYDVVPNHMN RGYPDKINL PAGQGFWRND CADPGNYPND CDDGDRFIGG
 DADLNTGHPQ VYGMFRDEFT NLRQYGAGG FRDFVRGYA PERVNSWMTD SADNSFCVGE
 LWKGPSEYPN WDWNTASWQ QIIKDWSRA KCPVDFDFAK ERMQNGSIAD WKHGLNGNP
 PRWREVAVTF VDNHDTGYSP QNGGQHWA LQDGLIRQAY AYILTSPGTP VVYWSHMYDW
 YGDFIRQLI QVRAAGVRA DSAISFHSGY SGLVATVSGS QTLVVALNS DLNPGQVAS

2250 GSFSEAVNAS NGQVRVWRSG TGSGGGEPGA LVSVSFRCDN GATQMGDSVY AVGNVSQLGN
WSPAAALRLT DTSGYPTWKG SIALPAGQNE EWKCLIRNEA NATQVRQWQG GANNSLTPSE
GATTVGRL

SEQ ID NO: 12

P.stutzeri maltotetraose-forming amylase (amyP) gene, complete cds. GenBank

2255 accession number M24516.

1 gatcggcctt tacggaaagt gatagagctt ctcttcgggc aaactttgtt cccagtgac
61 agaggggttag tatcggtatc cttcctcttt gggtttggtg gatcaggagc gccgagagca
121 ggatgaaatc ctgcgccagc aaggtcgcg cgaagatgtg gaactgctgc tggccgagat
181 ccggccggcg ttcattctcg tccggcgggc ttgccgccag ctaccggaac aagcacaaga
2260 241 accggagtat tgcgatgagc cacatcctgc gagccgcgtt attggcggtc atgctgttgc
301 cgttgccgtc catggccgat caggccggca agagcccaa cgctgtgcgc taccacggcg
361 gcgacgaaat cattctccag ggctttcact ggaacgtcgt ccgcaagcg cccaacgact
421 ggtacaacat cctgcgccag caggccgcga ccatcgccgc cgacggcttc tcggcgatct
481 ggatgccggt gccctggcgc gacttctcca gctggagcga cggcagcaag tccggcggtc
2265 541 gtgaaggcta cttctggcac gacttcaaca agaacggccg ctatggcagt gacgcccagc
601 tgcgtcaggc cgccagcgcg ctccgtggcg ccggcggtga agtgctttac gacgtggtgc
661 ccaaccacat gaaccgtggc tatccggaca aggagatcaa cctcccggtc ggccagggtc
721 tctggcgcaa cgactgcgcg gaccgggca actacccaa tgattgcgac gacggcgacc
781 gcttcatcgg cggcgatgcg gacctcaaca ccggccaccc gcaggtctac ggcatgttcc
2270 841 gcgatgaatt caccaacctg cgcagtcagt acgggtgcgg cggtctccgc ttcgactttg
901 ttccggggcta tgcgcgggag cgggtcaaca gctggatgac cgatagcgcc gacaacagct
961 tctgcgtcgg cgaactgtgg aaaggccctt ctgagtaccc gaactgggac tggcgcaaca
1021 ccgccagctg gcagcagatc atcaaggact ggtccgaccg ggccaagtgc ccggtgttcg
1081 acttcgccct caaggaacgc atgcagaacg ctcgatcgcc gactggaagc acgctgaac
2275 1141 ggcaatcccg acccgcggtg cgcgaggtgg cggtagcctt cgtcgacaac cacgacaccg
1201 gctactcgcc cgggcagaa cgggtggcagc accactgggc tctgcaggac gggctgatcc
1261 gccaggccta cgctacatc ctcaccagcc ccggtacgcc ggtggtgtac tggtcgcaca
1321 tgtacgactg ggttacggc gacttcatcc gtcagtgat ccaggtgctg ccgcccggc
1381 gcgtgcgcgc cgattcggtg atcagcttcc acagcggtc cagcggctg gtccccaccg
2280 1441 tcagcggcag ccagcagacc ctgggtggtg cgtcaactc cgacctgggc aatcccggcc
1501 aggtggccag cggcagcttc agcagggcgg tcaacgccag caacggccag gtgcgcgtgt
1561 ggcgtagcgg cacgggcagc ggtggcggtg aaccggcgcc tctggtcagt gtgagtttcc
1621 gctgcgacaa cggcgcgacg cagatgggag acagcgtcta cgcggtcgcc aacgtcagcc
1681 agctcggtaa ctggagcccc gcccgcggtg tgcgctgac cgacaccagc ggctaccgca
2285 1741 cctggaaggg cagcattgct ttgcttgcgg gccagaacga ggaatggaaa tgctgatcc
1801 gcaacgaggg caacgccacc taggtgcggc aatggcaggg cggggcaaac aacagcctga
1861 cgccgagcga gggcgccacc accgtcggtc ggctctagcc cgggcggcaa ctccggcgtc
1921 tcgcggtatg gaggcggctg gtctcgcgcg cggtagcgtt gcgctggggg cggggcgcc
1981 gtacacgcgc cctgctatcg ctagtcttcg gcgctccgag catcgccagc ttgccagcga
2290 2041 atcgccctgc cttcggcctg gtgcaggtcg tcgagcagcg ct

SEQ ID NO: 13

PSac-pPD77d33 sequence; Pseudomonas saccharophila maltotetrahydrolase amino acid

2295 sequence with 10 substitutions (N33Y, D34N, G134R, A141P, I157L, L178F, A179T, G223A, H307L, S334P) and deletion of the starch binding domain.

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG FSAIWMPPVPW
61 RDFSSWTDGG KSGGGEGYFW HDFNKNRGYR SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
121 GYPDKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFN

2300 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSDRAK CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 421 GDGGGNDGG

2305 SEQ ID NO: 14

PSac-D34(Y33N) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 10 substitutions and deletion of the starch binding domain.

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APNNWYNILR QQASTIAADG FSAIWMPVPW
 61 RDFSSWTDPG KSGGGEYFW HGFNKNGRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 2310 121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSDRAK CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 2315 421 GDGGGNDGG

SEQ ID NO: 15

PSac-D20(Y33N) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 12 substitutions and deletion of the starch binding domain.

2320 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APNNWYNILR QQASTIAADG FSAIWMPVPW
 61 RDFSSWTDPG RSGGGEYFW HGFNKNGRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSDRAK CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 2325 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGEFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 421 GDGGGNDGG

SEQ ID NO: 16

PSac-D14(Y33N) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 13 substitutions and deletion of the starch binding domain.

2330 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APNNWYNILR QQASTIAADG FSAIWMPVPW
 61 RDFSSWTDPG RSGGGEYFW HGFNKNSRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 2335 241 IIKDWSDRAK CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGEFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 421 GDGGGNDGG

2340 SEQ ID NO: 17

PStu-D34(Y33N) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 8 substitutions.

1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APNMWYNILR QQAATIAADG FSAIWMVPVPW
 61 RDFSSWSDPS KSGGGEYFW HDFNKNGRYG SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
 2345 121 DYPDKINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 241 IIKDWSRAK CPVDFDFAKE RMQNGSIADW KHGLNGNPDP RWREAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ VRRAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSQT
 2350 421 GSGGGEFGAL VSVSFRCDNG ATQMGSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

SEQ ID NO: 18

PStu-D20(Y33N) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid

2355 sequence with 10 substitutions.

1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APNMWYNILR QQAATIAADG FSAIWMVPVPW
 61 RDFSSWSDPS RSGGGEYFW HDFNKNGRYG SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
 121 DYPDKINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 2360 241 IIKDWSRAK CPVDFDFAKE RMQNGSIADW KHGLNGNPDP RWREAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ VRRAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSQT
 421 GSGGGEFGAL VSVSFRCDNG ATQMGSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL
 2365

SEQ ID NO: 19

PStu-D14(Y33N) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid

sequence with 11 substitutions.

1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APNMWYNILR QQAATIAADG FSAIWMVPVPW
 2370 61 RDFSSWSDPS RSGGGEYFW HDFNKNSRYG SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
 121 DYPDKINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 241 IIKDWSRAK CPVDFDFAKE RMQNGSIADW KHGLNGNPDP RWREAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ VRRAGVRAD
 2375 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSQT
 421 GSGGGEFGAL VSVSFRCDNG ATQMGSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

SEQ ID NO: 20

2380 PSac-pPD77d33(Y33N) sequence; *Pseudomonas saccharophila* maltotetrahydrolase
 amino acid sequence with 9 substitutions (D34N, G134R, A141P, I157L, L178F, A179T,
 G223A, H307L, S334P) and deletion of the starch binding domain.

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APNMWYNILR QQAATIAADG FSAIWMVPVPW
 2385 61 RDFSSWTDG KSGGGEYFW HDFNKNGRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 121 GYPDKINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 181 LRSYGAGGF RFDFVRYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSRAK CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREAVTFV DNHDTGYSPG

301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 2390 421 GDGGGNDGG

SEQ ID NO: 21

PSac-D34(N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 10 substitutions and deletion of the starch binding domain.

2395 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYDWYNILR QCASTIAADG FSAIWMPVPW
 61 RDFSSWTDPG KSGGGEYFW HDFNKNGRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 121 DYPDKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSRAK CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 2400 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 421 GDGGGNDGG

SEQ ID NO: 22

2405 PSac-D20(N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 12 substitutions and deletion of the starch binding domain.

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYDWYNILR QCASTIAADG FSAIWMPVPW
 61 RDFSSWTDPG RSGGGEYFW HDFNKNGRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 121 DYPDKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 2410 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSRAK CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGEFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 421 GDGGGNDGG

2415 SEQ ID NO: 23

PSac-D14(N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 13 substitutions and deletion of the starch binding domain.

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYDWYNILR QCASTIAADG FSAIWMPVPW
 61 RDFSSWTDPG RSGGGEYFW HDFNKNSRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 2420 121 DYPDKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSRAK CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGEFIRQLIQ VRRTAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 2425 421 GDGGGNDGG

SEQ ID NO: 24

PStu-D34(N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 8 substitutions.

2430 1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APYDWYNILR QQAATIAADG FSAIWMPVPW

61 RDFSSWSDPS KSGGGEYFW HDFNKNGRYG SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
 121 DYPDKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRGYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 241 IIKDWSDRAK CPVFDFALKE RMQNGSIADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 2435 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ VVRAAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSQT
 421 GSGGGEPPAL VSVSFRCDNG ATQMGSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

2440 SEQ ID NO: 25

PStu-D20(N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid
 sequence with 10 substitutions.

1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APYDWYNILR QQAATIAADG FSAIWMPVPW
 61 RDFSSWSDPS RSGGGEYFW HDFNKNGRYG SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
 2445 121 DYPDKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRGYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 241 IIKDWSDRAK CPVFDFALKE RMQNGSIADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ VVRAAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSQT
 2450 421 GSGGGEPPAL VSVSFRCDNG ATQMGSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

SEQ ID NO: 26

PStu-D14(N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid
 sequence with 11 substitutions.

1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APYDWYNILR QQAATIAADG FSAIWMPVPW
 61 RDFSSWSDPS RSGGGEYFW HDFNKNSRYG SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
 121 DYPDKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRGYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 2460 241 IIKDWSDRAK CPVFDFALKE RMQNGSIADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ VVRAAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSQT
 421 GSGGGEPPAL VSVSFRCDNG ATQMGSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL
 2465

SEQ ID NO: 27

PSac-pPD77d33(N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase
 amino acid sequence with 9 substitutions (N33Y, G134R, A141P, I157L, L178F, A179T,
 G223A, H307L, S334P) and deletion of the starch binding domain.

2470 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYDWYNILR QQASTIAADG FSAIWMPVPW
 61 RDFSSWTDGG KSGGGEYFW HDFNKNGRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 121 GYPDKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSDRAK CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 2475 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ VRRTAGVRAD

361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
421 GDGGGNDGG

SEQ ID NO: 28

2480 PSac-D34(Y33N-N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 9 substitutions and deletion of the starch binding domain.

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APNDWYNILR QCASTIAADG FSAIWMPVPW
61 RDFSSWTDPG KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
2485 181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
241 IIKDWSRAK CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ VRRTAGVRAD
361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
421 GDGGGNDGG

2490

SEQ ID NO: 29

PSac-D20(Y33N-N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 11 substitutions and deletion of the starch binding domain.

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APNDWYNILR QCASTIAADG FSAIWMPVPW
2495 61 RDFSSWTDPG RSGGGEGYFW HDFNKNGRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
241 IIKDWSRAK CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGEFIRQLIQ VRRTAGVRAD
2500 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
421 GDGGGNDGG

SEQ ID NO: 30

PSac-D14(Y33N-N34D) sequence; *Pseudomonas saccharophila* maltotetrahydrolase amino acid sequence with 12 substitutions and deletion of the starch binding domain.

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APNDWYNILR QCASTIAADG FSAIWMPVPW
61 RDFSSWTDPG RSGGGEGYFW HDFNKNSRYG SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
181 LRSGYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
2510 241 IIKDWSRAK CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGEFIRQLIQ VRRTAGVRAD
361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
421 GDGGGNDGG

2515 SEQ ID NO: 31

PStu-D34(Y33N-N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino acid sequence with 7 substitutions.

1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APNDWYNILR QQAATIAADG FSAIWMPVPW

2520 61 RDFSSWSDPS KSGGGEYFW HDFNKNRGY SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
 121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRGYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 241 IIKDWSRAK CPVFDFALKE RMQNGSIADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ VVRAAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSRG
 2525 421 GSGGGEPPAL VSVSFRCDNG ATQMGDSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

SEQ ID NO: 32

PStu-D20(Y33N-N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino

2530 acid sequence with 9 substitutions.

1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APNDWYNILR QQAATIAADG FSAIWMPVPW
 61 RDFSSWSDPS RSGGGEYFW HDFNKNRGY SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
 121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRGYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 2535 241 IIKDWSRAK CPVFDFALKE RMQNGSIADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ VVRAAGVRAD
 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSRG
 421 GSGGGEPPAL VSVSFRCDNG ATQMGDSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

2540

SEQ ID NO: 33

PStu-D14(Y33N-N34D) sequence; *Pseudomonas stutzeri* maltotetrahydrolase amino

acid sequence with 10 substitutions.

1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APNDWYNILR QQAATIAADG FSAIWMPVPW
 2545 61 RDFSSWSDPS RSGGGEYFW HDFNKNRYG SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
 121 DYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
 181 LRSQYGAGGF RFDFVRGYAP ERVNSWMTDS ADNSFCVGEL WKAPSEYPNW DWRNTASWQQ
 241 IIKDWSRAK CPVFDFALKE RMQNGSIADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ VVRAAGVRAD
 2550 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRSRG
 421 GSGGGEPPAL VSVSFRCDNG ATQMGDSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
 481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

SEQ ID NO: 34

2555 PSac-pPD77d33(Y33N-N34D) sequence; *Pseudomonas saccharophila*

maltotetrahydrolase amino acid sequence with 8 substitutions (G134R, A141P, I157L, L178F, A179T, G223A, H307L, S334P) and deletion of the starch binding domain.

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APNDWYNILR QQAATIAADG FSAIWMPVPW
 2560 61 RDFSSWTDGG KSGGGEYFW HDFNKNRGY SDAQLRQAAG ALGGAGVKVL YDVVPNHMNR
 121 GYPDKKEINLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGE SDLNTGHPQI YGMFRDEFTN
 181 LRSYGAGGF RFDFVRGYAP ERVDSWMSDS ADSSFCVGEL WKAPSEYPSW DWRNTASWQQ
 241 IIKDWSRAK CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ VVRTAGVRAD

361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG SFSEAVNASN GQVRVWRSGS
 2565 421 GDGGGNDGG

OTHER SEQUENCES

Other PS4 variant polypeptides, which are furthermore suitable for use as parent

2570 polypeptides, are set out below:

| | | | | | | | |
|------|-----|------------|------------|------------|-------------|------------|------------|
| | 1 | DQAGKSPAGV | RYHGGDEIIL | QGFHWNVRE | APYNWYNILR | QASTIAADG | FSAIWMPVPW |
| | 61 | RDFSSWTDPG | KSGGGEYFW | HDFNKNGRYG | SDAQLRQAAG | ALGGAGVKVL | YDVVPNHMNR |
| | 121 | GYPDKEINLP | AGQRFWRNDC | PDPGNYPNDC | DDGDRFLGGE | SDLNTGHPQI | YGMFRDEFTN |
| | 181 | LRSYGAGGF | RFDFVRGYAP | ERVDSWMSDS | ADSSFCVGEL | WKAPSEYPSW | DWRNTASWQQ |
| 2575 | 241 | IIKDWSRAK | CPVDFDFAKE | RMQNGSVADW | KHGLNGNPDP | RWREVAVTFV | DNHDTGYSPG |
| | 301 | QNGGQHLWAL | QDGLIRQAYA | YILTSPGTPV | VYWPAMYDWG | YGDFIRQLIQ | VRRTAGVRAD |
| | 361 | SAISFHSGYS | GLVATVSGSQ | QTLVVALNSD | LANPGQVASG | SFSEAVNASN | GQVRVWRSGS |
| | 421 | GDGGGNDGGE | GGLVNVNFR | DNGVTQMGDS | VYAVGNVSQL | GNWSPASAVR | LTDTSSTPTW |
| | 481 | KGSIALPDGQ | NVEWKCLIRN | EADATLVRQW | QSGGNNQVQA | AAGASTSGSF | |
| 2580 | 1 | DQAGKSPAGV | RYHGGDEIIL | QGFHWNVRE | APYNWYNILR | QASTIAADG | FSAIWMPVPW |
| | 61 | RDFSSWTDPG | KSGGGEYFW | HDFNKNGRYG | SDAQLRQAAG | ALGGAGVKVL | YDVVPNHMNR |
| | 121 | DYPDKEINLP | AGQRFWRNDC | PDPGNYPNDC | DDGDRFLGGE | SDLNTGHPQI | YGMFRDEFTN |
| | 181 | LRSYGAGGF | RFDFVRGYAP | ERVDSWMSDS | ADSSFCVGEL | WKAPSEYPSW | DWRNTASWQQ |
| 2585 | 241 | IIKDWSRAK | CPVDFDFAKE | RMQNGSVADW | KHGLNGNPDP | RWREVAVTFV | DNHDTGYSPG |
| | 301 | QNGGQHLWAL | QDGLIRQAYA | YILTSPGTPV | VYWPAMYDWG | YGDFIRQLIQ | VRRTAGVRAD |
| | 361 | SAISFHSGYS | GLVATVSGSQ | QTLVVALNSD | LANPGQVASG | SFSEAVNASN | GQVRVWRSGS |
| | 421 | GDGGGNDGGE | GGLVNVNFR | DNGVTQMGDS | VYAVGNVSQL | GNWSPASAVR | LTDTSSTPTW |
| 2590 | 1 | DQAGKSPAGV | RYHGGDEIIL | QGFHWNVRE | APYNWYNILR | QASTIAADG | FSAIWMPVPW |
| | 61 | RDFSSWTDPG | KSGGGEYFW | HDFNKNSRYG | SDAQLRQAAG | ALGGAGVKVL | YDVVPNHMNR |
| | 121 | DYPDKEINLP | AGQRFWRNDC | PDPGNYPNDC | DDGDRFLGGE | SDLNTGHPQI | YGMFRDEFTN |
| | 181 | LRSYGAGGF | RFDFVRGYAP | ERVDSWMSDS | ADSSFCVGEL | WKAPSEYPSW | DWRNTASWQQ |
| | 241 | IIKDWSRAK | CPVDFDFAKE | RMQNGSVADW | KHGLNGNPDP | RWREVAVTFV | DNHDTGYSPG |
| 2595 | 301 | QNGGQHLWAL | QDGLIRQAYA | YILTSPGTPV | VYWPAMYDWG | YGDFIRQLIQ | VRRTAGVRAD |
| | 361 | SAISFHSGYS | GLVATVSGSQ | QTLVVALNSD | LANPGQVASG | SFSEAVNASN | GQVRVWRSGS |
| | 421 | GDGGGNDGGE | GGLVNVNFR | DNGVTQMGDS | VYAVGNVSQL | GNWSPASAVR | LTDTSSTPTW |
| 2600 | 1 | DQAGKSPNAV | RYHGGDEIIL | QGFHWNVRE | APYNWYNILR | QAATIAADG | FSAIWMPVPW |
| | 61 | RDFSSWSDPS | KSGGGEYFW | HDFNKNGRYG | SDAQLRQAAS | ALGGAGVKVL | YDVVPNHMNR |
| | 121 | GYPDKEINLP | AGQRFWRNDC | PDPGNYPNDC | DDGDRFLGGD | ADLNTGHPQV | YGMFRDEFTN |
| | 181 | LRSYGAGGF | RFDFVRGYAP | ERVNSWMTDS | ADNSFCVGEL | WKAPSEYPNW | DWRNTASWQQ |
| | 241 | IIKDWSRAK | CPVDFDFAKE | RMQNGSIADW | KHGLNGNPDP | RWREVAVTFV | DNHDTGYSPG |
| | 301 | QNGGQHLWAL | QDGLIRQAYA | YILTSPGTPV | VYWPAMYDWG | YGDFIRQLIQ | VRRAAGVRAD |
| 2605 | 361 | SAISFHSGYS | GLVATVSGSQ | QTLVVALNSD | LGPNPGQVASG | SFSEAVNASN | GQVRVWRSGT |
| | 421 | GSGGGEPPAL | VSVSFRCDNG | ATQMGDSVYA | VGNVSQLGNW | SPAAALRLTD | TSGYPTWKGS |
| | 481 | IAPAGQNEE | WKCLIRNEAN | ATQVRQWQGG | ANNSLTPSEG | ATTVGRL | |
| 2610 | 1 | DQAGKSPNAV | RYHGGDEIIL | QGFHWNVRE | APYNWYNILR | QAATIAADG | FSAIWMPVPW |
| | 61 | RDFSSWSDPS | KSGGGEYFW | HDFNKNGRYG | SDAQLRQAAS | ALGGAGVKVL | YDVVPNHMNR |
| | 121 | DYPDKEINLP | AGQRFWRNDC | PDPGNYPNDC | DDGDRFLGGD | ADLNTGHPQV | YGMFRDEFTN |
| | 181 | LRSYGAGGF | RFDFVRGYAP | ERVNSWMTDS | ADNSFCVGEL | WKAPSEYPNW | DWRNTASWQQ |
| | 241 | IIKDWSRAK | CPVDFDFAKE | RMQNGSIADW | KHGLNGNPDP | RWREVAVTFV | DNHDTGYSPG |
| | 301 | QNGGQHLWAL | QDGLIRQAYA | YILTSPGTPV | VYWPAMYDWG | YGDFIRQLIQ | VRRAAGVRAD |

2615 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRS GT
421 GSGGGEPPAL VSVSFRCDNG ATQMGDSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

1 DQAGKSPNAV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQAATIAADG FSAIWMPVPW
2620 61 RDFSSWSDPS KSGGGEYFW HDFNKNSRYG SDAQLRQAAS ALGGAGVKVL YDVVPNHMNR
121 DYPDKELNLP AGQRFWRNDC PDPGNYPNDC DDGDRFLGGD ADLNTGHPQV YGMFRDEFTN
181 LRSQYGAGGF RFDVVRGYAP ERVNSWMTDS ADNSFCV GEL WKAPSEYPNW DWRNTASWQQ
241 I IKDWS DR AK CPVFDFALKE RMQNGSIADW KHGLNGNPD P RWREVA VTFV DNHDTGYSPG
301 QNGGQHLWAL QDGLIRQAYA YILTSPGTPV VYWP MYDWG YGDFIRQLIQ VRRAGVRAD
2625 361 SAISFHSGYS GLVATVSGSQ QTLVVALNSD LGNPGQVASG SFSEAVNASN GQVRVWRS GT
421 GSGGGEPPAL VSVSFRCDNG ATQMGDSVYA VGNVSQLGNW SPAAALRLTD TSGYPTWKGS
481 IALPAGQNEE WKCLIRNEAN ATQVRQWQGG ANNSLTPSEG ATTVGRL

CLAIMS

1. A food additive comprising a PS4 variant polypeptide, in which the PS4 variant polypeptide is derivable from a parent polypeptide having non-maltogenic exoamylase activity, in which the PS4 variant polypeptide comprises substitutions at the following positions: 134, 141, 157, 223, 307 and 334 with reference to the position numbering of a *Pseudomonas saccharophilia* exoamylase sequence shown as SEQ ID NO: 1.
2. A food additive according to Claim 1, further comprising one or both of substitutions at positions 121 and 223, preferably G121D and/or G223A.
3. A food additive according to Claim 1 or Claim 2, further comprising one or more of: a substitution at position 33, preferably N33, more preferably N33Y, a substitution at position 34, preferably D34, more preferably D34N, a substitution at position 178 and a substitution at position 179.
4. A food additive according to Claim 1 or 2, in which the parent polypeptide comprises a non-maltogenic exoamylase, preferably a glucan 1,4-alpha-maltotetrahydrolase (EC 3.2.1.60), more preferably being derivable from *Pseudomonas* species, preferably *Pseudomonas saccharophilia* or *Pseudomonas stutzeri*.
5. A food additive according to any preceding claim, in which the parent polypeptide is a non-maltogenic exoamylase from *Pseudomonas saccharophilia* exoamylase having a sequence shown as SEQ ID NO: 1 or SEQ ID NO: 5.
6. A food additive according to any of Claims 1 to 7, in which the parent polypeptide is a non-maltogenic exoamylase from *Pseudomonas stutzeri* having a sequence shown as SEQ ID NO: 7 or SEQ ID NO: 11.
7. A food additive according to any preceding claim, which has a higher thermostability compared to the parent polypeptide when tested under the same conditions.
8. A food additive according to any preceding claim, in which the half life ($t_{1/2}$), preferably at 60 degrees C, is increased by 15% or more, preferably 50% or more, most preferably 100% or more, relative to the parent polypeptide.

9. A food additive according to any preceding claim, which has a higher exo-specificity compared to the parent polypeptide when tested under the same conditions.
10. A food additive according to any preceding claim, which has 10% or more, preferably 20% or more, preferably 50% or more, exo-specificity compared to the parent polypeptide.
- 2660 11. A food additive according to any preceding claim, in which the position 134 substitution comprises G134R.
12. A food additive according to any preceding claim, in which the position 141 substitution comprises A141P.
- 2665 13. A food additive to any preceding claim, in which the position 334 substitution comprises S334P.
14. A food additive according to any preceding claim, in which
- (a) the position 33 substitution comprises N33Y;
- (b) the position 34 substitution comprises D34N;
- 2670 (c) the position 157 substitution comprises I157L;
- (d) the position 178 substitution comprises L178F;
- (e) the position 179 substitution comprises A179T.
- (f) the position 223 substitution comprises G223A; or
- (g) the position 307 substitution comprises H307L.
- 2675 15. A food additive according to any preceding claim, which comprises the substitutions: G134R, A141P, I157L, G223A, H307L and S334P, together with phenylalanine at position 178 or threonine at position 179, or both, optionally together with N33Y and D34N.

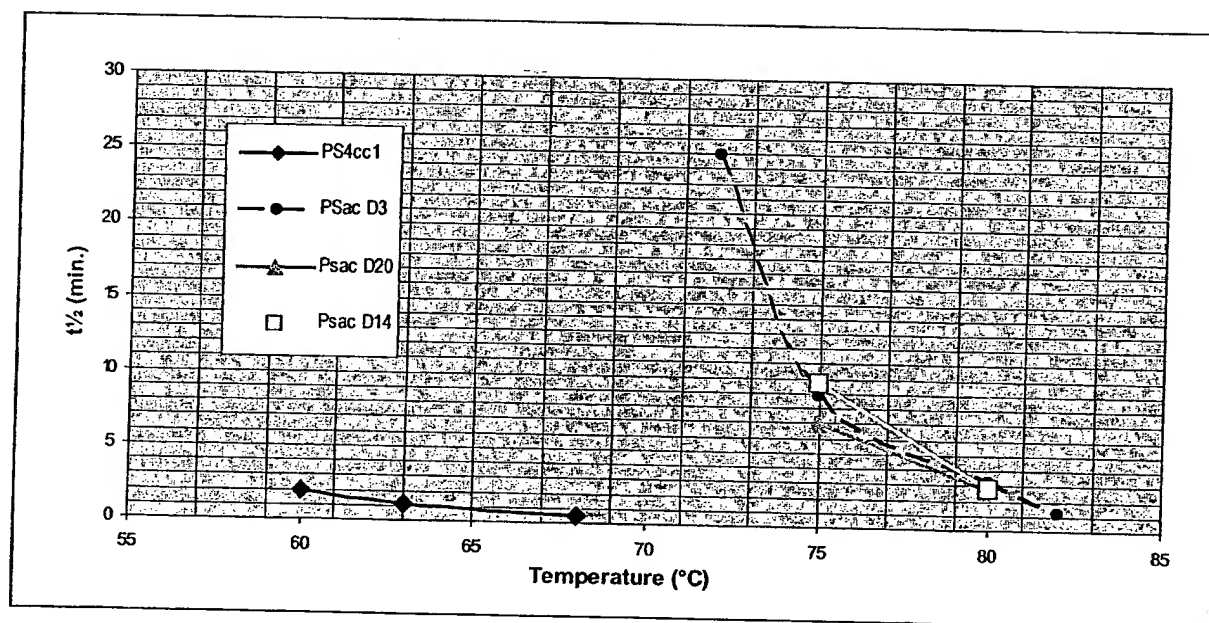
16. A food additive according to any preceding claim, which has the sequence PSac-
2680 D34 (SEQ ID NO: 2) or the sequence PStu-D34 (SEQ ID NO: 8).
17. A food additive according to any preceding claim, which further comprises a substitution at position 121, preferably G121D.
18. A food additive according to Claim 17, which has the sequence PSac-D20 (SEQ ID NO: 3) or the sequence PStu-D20 (SEQ ID NO: 9).
- 2685 19. A food additive according to any preceding claim, which further comprises a substitution at position 87, preferably G87S.
20. A food additive according to Claim 19, which has the sequence PSac-D14 (SEQ ID NO: 4).
21. A food additive according to Claim 19, which has the sequence PStu-D14 (SEQ ID
2690 NO: 10).
22. A food additive according to any preceding claim, which has the sequence PSac-pPD77d33.
23. Use of a PS4 variant polypeptide as set out in any preceding claim as a food additive.
- 2695 24. A process for treating a starch comprising contacting the starch with a PS4 variant polypeptide as set out in any preceding claim and allowing the polypeptide to generate from the starch one or more linear products.
25. Use of a PS4 variant polypeptide as set out in any of Claims 1 to 23 in preparing a
2700 food product.
26. A process of preparing a food product comprising admixing a polypeptide as set out in any of Claims 1 to 23 with a food ingredient.
27. Use according to Claim 25, or a process according to Claim 26, in which the food product comprises a dough or a dough product, preferably a processed dough product.

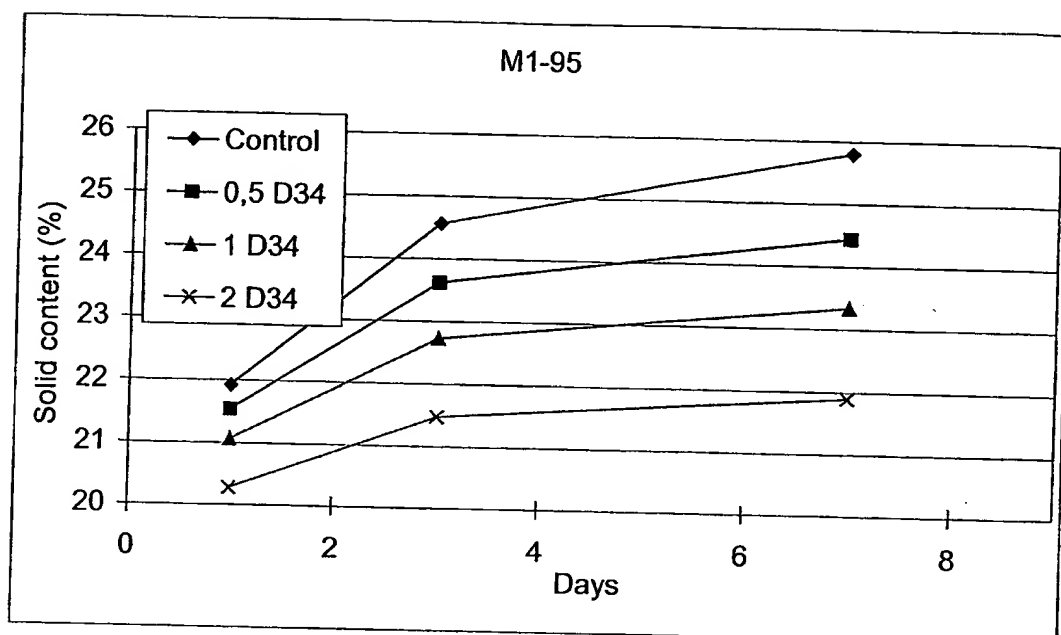
- 2705 28. A use or process according to any of Claims 25 to 7, in which the food product is a bakery product.
29. A process for making a bakery product comprising: (a) providing a starch medium; (b) adding to the starch medium a PS4 variant polypeptide as set out in any of Claims 1 to 23; and (c) applying heat to the starch medium during or after step (b) to produce a bakery
2710 product.
30. A food product, dough product or a bakery product obtained by a process according to any of Claims 25 to 29.
31. An improver composition for a dough, in which the improver composition comprises a PS4 variant polypeptide as set out in any of Claims 1 to 23, and at least one
2715 further dough ingredient or dough additive.
32. A composition comprising a flour and a PS4 variant polypeptide as set out in any of Claims 1 to 23.
33. Use of a PS4 variant polypeptide as set out in any of Claims 1 to 23, in a dough product to retard or reduce staling, preferably detrimental retrogradation, of the dough
2720 product.
34. A combination of a PS4 variant polypeptide as set out in any preceding claim, together with Novamyl, or a variant, homologue, or mutants thereof which has maltogenic alpha-amylase activity.
35. Use of a combination according to Claim 34 for an application according to any
2725 preceding claim.
36. A food product produced by treatment with a combination according to Claim 34.
37. A food additive comprising a PS4 variant polypeptide, in which the PS4 variant polypeptide is derivable from a parent polypeptide having non-maltogenic exoamylase activity, in which the PS4 variant polypeptide comprises substitutions at the following
2730 positions: G121D, 134, 141, 157, 223, 307 and 334 with reference to the position

numbering of a *Pseudomonas saccharophila* exoamylase sequence shown as SEQ ID NO: 1.

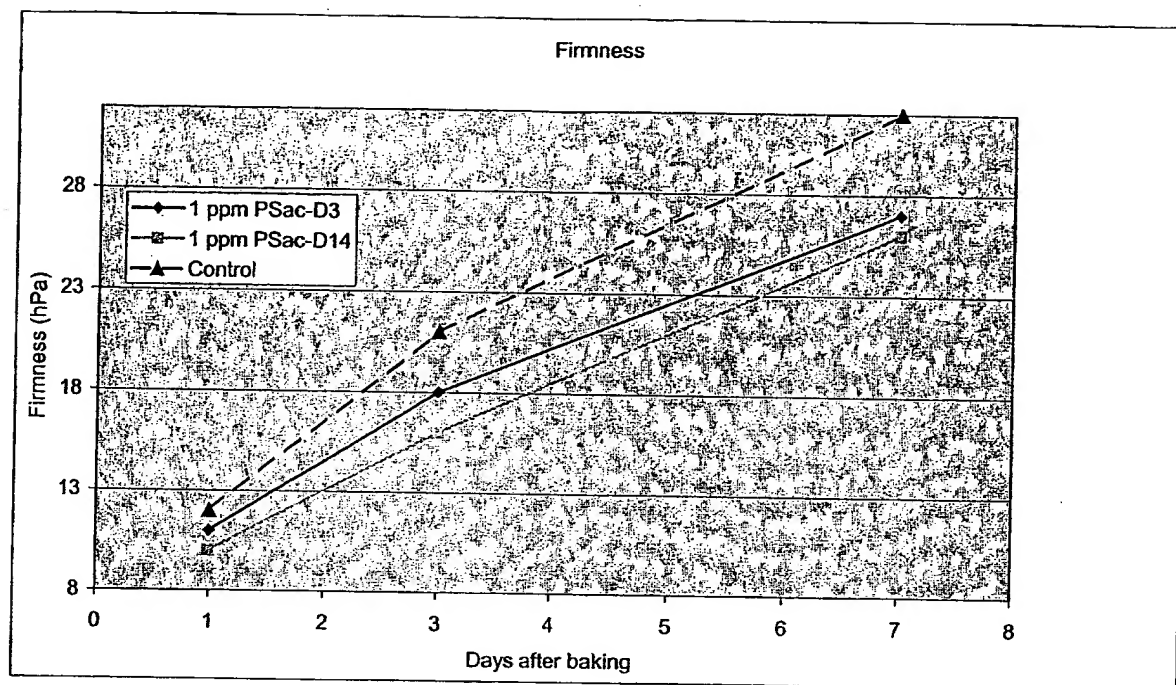
38. Use of a PS4 variant polypeptide substantially as hereinbefore described with reference to and as shown in the accompanying drawings.
- 2735 39. A combination comprising a PS4 nucleic acid substantially as hereinbefore described with reference to and as shown in the accompanying drawings.

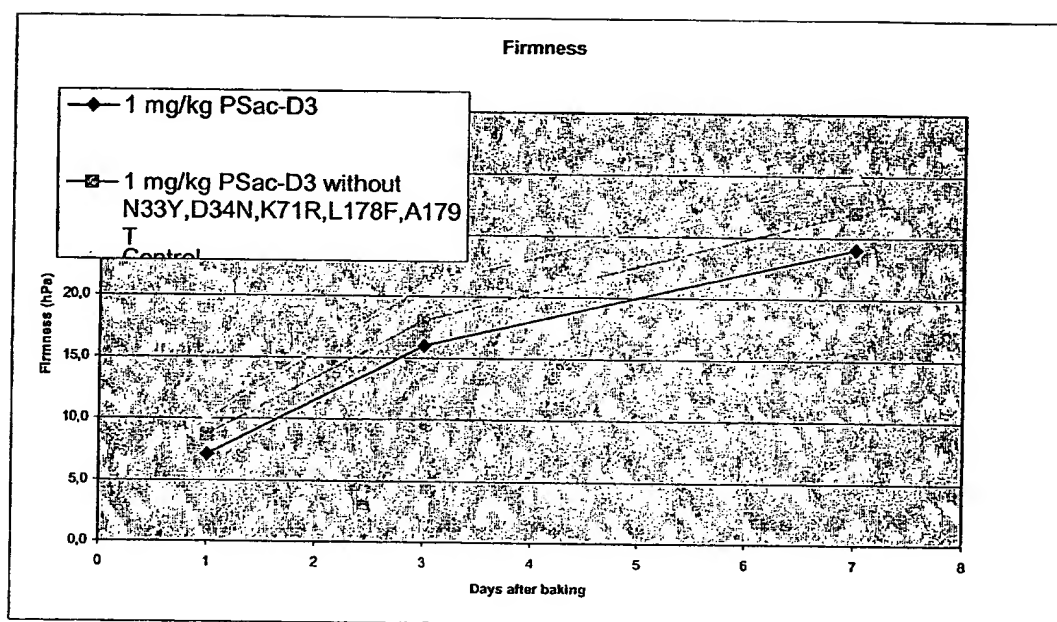
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**FIGURE 1**

**FIGURE 2**

3/4

**FIGURE 3**

**FIGURE 4**

INTERNATIONAL SEARCH REPORT

International Application No

IB2004/002487

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/28 A23L1/03 A21D8/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, Sequence Search, BIOSIS

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T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

23 November 2004

Date of mailing of the international search report

06/12/2004

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INTERNATIONAL SEARCH REPORT

International Application No

IB2004/002487

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